Volume 16 SEPTEMBER-OCTOBER, 1951 Number 5

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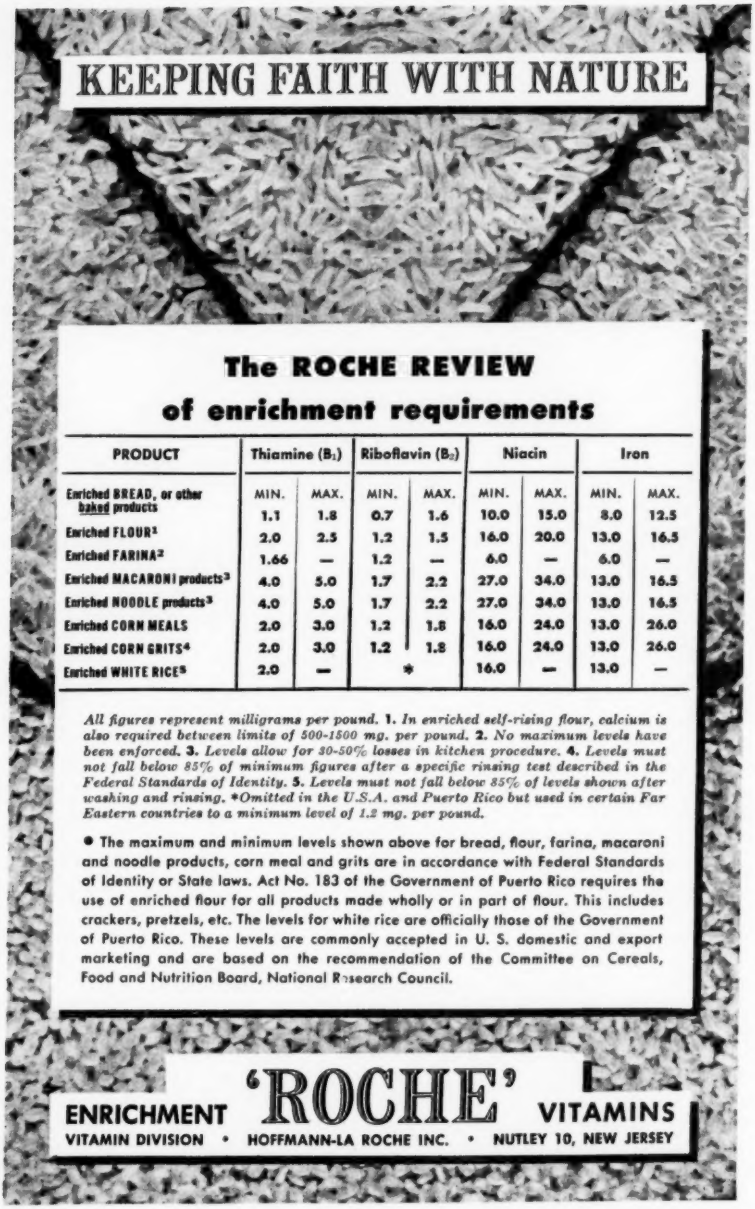

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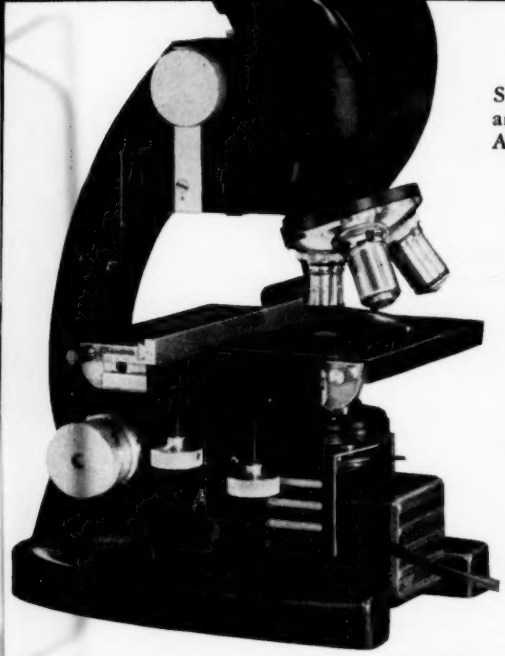

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EFFECT OF ANTIOXIDANTS ON ORANGE OIL

E. M. KENYON[a] AND B. E. PROCTOR
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(Manuscript received September 28, 1950)

The losses incurred in the air oxidation of citrus oils during storage and handling constitute a serious problem to the essential oil industry. The end result of such oxidation is the development of "off" odors and flavors in these oils, which lower or destroy entirely their market value. The probability has been pointed out previously *(27)* that air oxidation, at least in part, may result in double bond oxidation and further breakdown, to yield compounds with undesirable odors. In this respect, oxidation of citrus oils is similar to autoxidation in unsaturated fats and oils.

As a step toward the development of methods to prevent the deleterious effects of oxidation of citrus oils, an investigation was made to determine whether antioxidants such as are used in edible fats and oils might retard autoxidation in citrus oils.

THEORETICAL ASPECTS OF ANTIOXIDANTS

The existence, in fats and oils, of natural substances that delay the reaction between the substances being oxidized and the oxygen of the air has been known for some time. Such materials occur in natural fats and oils in extremely minute amounts, representing from a few hundredths to even thousandths of one percent of the total amount of the fat or oil. Probably the first mention of such substances was made more than one hundred years ago by Deschamps *(6)*, who observed that lard containing vanillin or saligenin did not become rancid so quickly as lard without these substances. In 1926 and in 1928, Moureu and Dufraisse *(22, 23)* studied the effect of a number of substances on the velocity of oxidative reactions. They found that many of the substances studied delayed the reaction with oxygen. Such materials have been variously termed antioxygens, inhibitors, and antioxidants.

Since this early work, considerable study has been devoted to antioxidants, and the list of these materials that are currently being used or that have been proposed for use is constantly growing. Lundberg *(17)* lists forty-two such materials, several of which are groups containing a number of individual compounds.

Early in the research on antioxidants, Christiansen *(5)* proposed a theory concerning the mechanism of antioxidants. Application of his theoretical approach makes it appear that a molecule of fat or oil can form peroxides by reaction with oxygen only after one or both are activated by the absorption of a quantum of energy. This energy may have its source in some chemical compound that is present which has been previously acti-

[a] Present address: Office of the Quartermaster General, Washington, D. C.

vated or it may come from external sources, such as light or heat. After the peroxide has been formed, it may release its energy, which can then serve to activate additional molecules with the resultant formation of new peroxides. Thus a chain is established, which will continue unless the energy of activation can be absorbed in side reactions. It is proposed that antioxidants absorb this energy, breaking the chain and becoming oxidized themselves. This theory of the function of antioxidants is essentially that held today *(11, 19)*.

The inhibition of autoxidation in fats and oils has been attributed to two types of substances classified by Mattill *(20)* as antioxidants and synergists. With few exceptions, the antioxidants for fats are ortho- and para-diphenolic and polyphenolic compounds or materials having a similar electronic configuration. The synergists are usually di- and poly-basic acids, which appear to reinforce the effect of the phenolic type of compound instead of acting directly. Only the first type of compound or the true antioxidant will be considered here. It is possible, however, that future investigation will indicate that synergists of value for fats and oils may be useful for essential oils as well.

Lakritz *(14)* has described some work with antioxidants in orange and lemon oils. Although his results indicate a preservative action by the compounds he tested, Lakritz appears to have based his findings on organoleptic observations rather than on physical or chemical methods.

EXPERIMENTAL PROCEDURE

To study the effect of antioxidants on orange oil, the Warburg method *(7, 31)* of following oxidation or oxygen absorption was employed. This method has been shown to be a means of measuring odor deterioration *(27)*. The temperature at which the oxidations were carried out, the rate of shaking of the reaction flasks, the weights of the samples, and the technique of procedure were identical with those described elsewhere *(27)*.

Five antioxidants were studied, namely, alpha-tocopherol, nordihydroguaiaretic acid, ethyl and propyl gallate, and guaiacol. These were added in amounts representing 0.05% by weight to samples of fresh commercial orange oil, which were then subjected to controlled oxidation by means of the Warburg system.

Antioxidants Used

Tocopherol. In early studies by Mattill and his coworkers *(4, 25, 26)* dealing with materials that inhibit the development of rancidity in vegetable oils, concentrates were isolated that proved to have antioxidant activity. Later, when tocopherols had been isolated *(9, 10)* and their antioxidant properties demonstrated *(24)*, it was recognized that the tocopherol content of these early concentrates was responsible for their activity. Several patents have since been issued in England and the United States to cover the use of these materials as antioxidants *(8, 30)*.

Methods for the preparation of fairly pure concentrates of tocopherol include the use of molecular distillation and fractional crystallization. Natural vegetable oils such as peanut oil are used as sources of tocopherols.

Tocopherols are found in many natural food materials, and it is reasonable to expect that orange oil contains significant concentrations of tocopherols. That some substance inhibiting the uptake of oxygen is present in orange oil is known from preliminary studies carried out in the course of the present investigation. Fairly pure limonene (the major constituent of orange oil) has an extremely rapid rate of oxidation with practically no induction period (initial period of low rate of oxygen uptake) as compared with the oil itself. This lack of an induction period and a rapid oxygen uptake may occur in fats and oils. Highly purified fats show no induction period or

almost none as compared with those containing small amounts of naturally occurring substances. As mentioned above, these substances appear to be or are related to tocopherols.

It is possible that the inhibiting substances present in orange oil may be tocopherols. In this case, the use of tocopherols as antioxidants would be the addition of an already naturally occurring substance, which is an advantage in a food material. Although tocopherol has not yet been used as an antioxidant, its use has been authorized in rendered animal fats in concentrations not exceeding 0.03% by weight.

Several investigators have studied tocopherol and its effectiveness as an antioxidant, including Mitchell and Black *(21)* and György and Tomarelli *(12)*. Tocopherol has been found to be fairly effective, even in small concentrations.

Nordihydroguaiaretic acid (NDGA). Lundberg, Halvorson, and Burr *(18)* were the first to demonstrate the antioxidant properties of this substance. A public service patent by Lauer *(15)*, assigned to the U. S. Department of Agriculture, covers its use as an antioxidant. During the Second World War, its use in fats up to a concentration of 0.01% by weight was approved by the War Food Administration, Meat Inspection Division.

Several investigators *(1, 13, 28)* have studied NDGA in various fat and oil systems, with and without synergistic compounds, and have shown it to be extremely effective in animal fats at a concentration of 0.01% by weight. This material appears to be somewhat less effective with vegetable fats.

From the standpoint of its use in food materials and essential oils in particular, NDGA apparently imparts no undesirable off odors, colors, or flavors to materials to which it is added.

NDGA is obtained by extraction from the plant *Larrea divaricata*. It is commonly known as the creosote bush and grows extensively in the desert regions of the southwestern United States and Mexico. Although the cost of the substance is high, so little of it is required that its addition to a product does not greatly increase its cost.

Ethyl and propyl gallate. These two materials will be discussed together as they are both similar esters of gallic acid. British investigators were the first to point out the use of propyl gallate as an antioxidant of the phenolic type *(2, 3)*. Other esters of gallic acid, such as ethyl gallate, have been studied by Lea *(16)*. He has indicated that propyl gallate did not appear toxic to mice and that no skin irritation resulted from the substance in the case of rabbits and humans. Propyl gallate is fairly soluble in food materials, is said to be heat stable, and is available in fairly pure condition in commercial amounts. It is reported to have no effect in causing added odors, colors, or flavors in fats and oils.

The use of propyl gallate as an antioxidant is covered by a United States patent, which deals with the lower chain alcohol esters of gallic acid *(29)*. Propyl gallate in concentrations up to 0.01% by weight has been approved for fats by the Meat Inspection Division of the War Food Administration. Hence it was thought desirable to study the effect of these compounds in orange oil. It was hoped that the use of both ethyl and propyl esters might give some information on the effect of varying alkyl chains on the antioxidant activity of these compounds.

Guaiacol. Guaiacol is the monomethyl ether of catechol and is found in beech wood tar. Catechol is O-dihydroxy-benzene. Guaiacol has not been used as an antioxidant in food materials, presumably because of its toxicity, but as it was available, its effect on orange oil was studied as being of theoretical interest.

RESULTS AND DISCUSSION

The results obtained with samples of orange oil treated with the antioxidants described above and subjected to controlled oxidation by the Warburg method are shown graphically in Figures 1 to 5.

As can be seen from Figure 1, alpha-tocopherol was effective in retarding oxygen absorption by orange oil at least up to 160 hours and probably beyond. According to the curve as drawn for the treated oil, an increase in oxygen absorption began at approximately 90 hours. However, the rate of oxygen absorption beyond 90 hours was still not particularly high, and

it is interesting to speculate whether a higher rate might be obtained beyond 160 hours. As it was the purpose of the present study to investigate the general question of whether antioxidants useful in preventing oxidation in edible fats and oils might be effective for the same purpose with essential oils, the run was not carried beyond 160 hours.

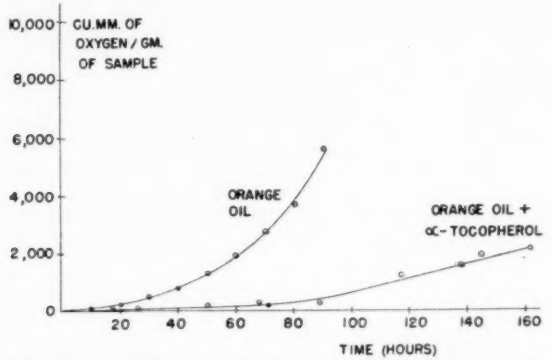

FIGURE 1. Effect of alpha-tocopherol on the oxygen absorption of orange oil at 40°C.

It is apparent from Figure 2 that NDGA almost doubled the induction period of the treated as compared with that of the untreated oil. That section of the curve indicating a more rapid rate of oxygen absorption may be a part of the induction period, but it is believed that this is unlikely in this case.

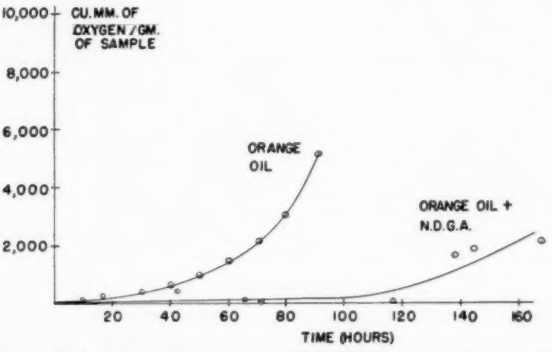

FIGURE 2. Effect of nordihydroguaiaretic acid (NDGA) on the oxygen absorption of orange oil at 40°C.

Comparison of the curves in Figures 3 and 4 shows that propyl gallate was somewhat more effective than ethyl gallate as an inhibitor of oxidation in orange oil.

Both of these antioxidants approximately doubled the induction period of the treated oil as compared with that of the untreated oil. As already pointed out, NDGA also almost doubled the induction period for the treated sample. However, comparison of the curves for the samples treated with the gallic acid derivatives and NDGA indicates that the rates of oxygen

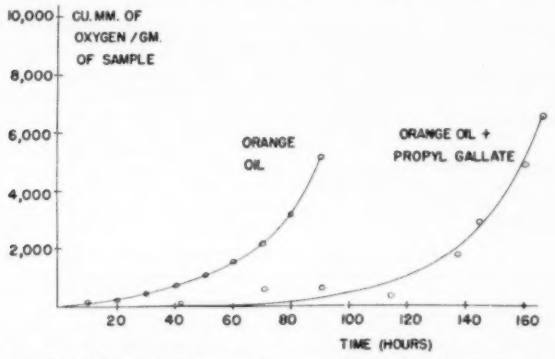

FIGURE 3. Effect of propyl gallate on the oxygen absorption of orange oil at 40°C.

absorption by the samples treated with ethyl and propyl gallate had definitely become more rapid at approximately 140 hours, whereas the rate of oxygen absorption by the sample treated with NDGA had only begun to increase at that time. It may be concluded that ethyl gallate and propyl gallate are both less effective antioxidants for orange oil than is NDGA.

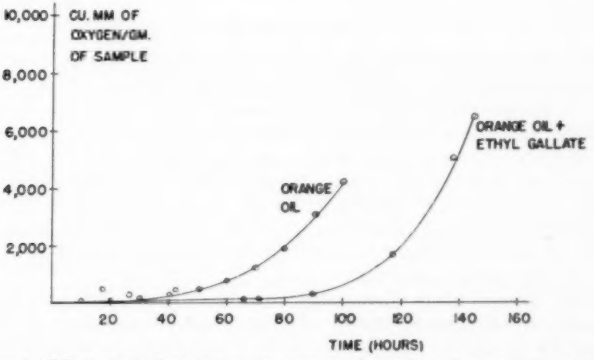

FIGURE 4. Effect of ethyl gallate on the oxygen absorption of orange oil at 40°C.

Guaiacol was even more effective in suppressing oxygen absorption than either alpha-tocopherol or NDGA (compare Figures 1, 2, and 5), which were the most potent of the other antioxidants studied.

At the conclusion of the several tests (after approximately 160 hours), no "off" odors were detectable in the samples treated with alpha-tocopherol, NDGA, and the gallic acid derivatives. At the beginning of the test with guaiacol, no odor due to this substance was detected in the treated sample. Organoleptic examination of this treated sample at the end of the test, however, showed that it had a marked foreign odor, reminiscent of the odor of guaiacol. This odor, together with the presumed toxicity, would probably exclude the use of guaiacol as an antioxidant for orange oil, unless future investigation should prove that lower concentrations of this substance are effective in prevention of oxidation and that the substance is not toxic to humans.

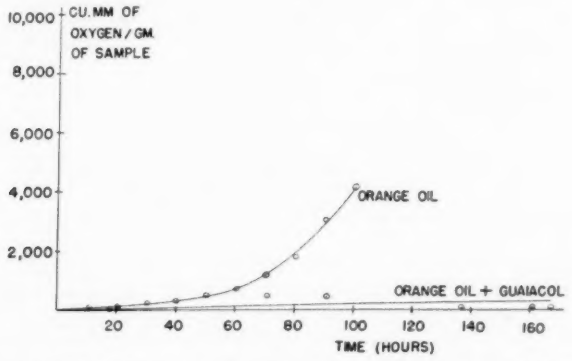

FIGURE 5. Effect of guaiacol on the oxygen absorption of orange oil at 40°C.

CONCLUSIONS

The experiments described in this report have shown that, as a class, those antioxidants that are effective in retarding oxidative changes in edible fats and oils are also effective for the same purpose in orange oil.

Of the five antioxidants studied, alpha-tocopherol and nordihydroguaiaretic acid (NDGA) appeared to offer the most promise for use with orange oil and possibly other citrus oils. Propyl gallate was more effective as an antioxidant than ethyl gallate, but both were less effective than alpha-tocopherol and NDGA. Guaiacol, although effective, should probably not be used as an antioxidant for orange oil, because of its strong characteristic odor and its possible toxicity.

It is believed that alpha-tocopherol offers particular promise as an inhibitor of oxidation in orange oil, for it may conceivably be a normal constituent of this oil and it appears to be especially effective in delaying autoxidation in orange oil.

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EFFECT OF TEMPERATURE OF STORAGE ON MICROBIAL CHANGES IN LIVER SAUSAGE AND BOLOGNA[a]

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In previous reports *(1, 2)* we have shown that commercial braunschweiger liver sausage stuffed in Visking and covered with a single layer of Pliofilm undergoes considerable microbial change on extended storage at 5°C.(41°F.). Organisms grew both in the sausage near the coverings and as a slime between the casings, although the types of bacteria in these two places were different. Similar sausage in single layers of Pliofilm or Saran showed no microbial change when refrigerated for as long as 11 weeks. On the basis of the observations, Saran appeared to be the preferable casing material because it not only prevented growth of organisms in the sausage but also prevented discoloration caused by absorption of oxygen. Under the conditions of storage liver sausage cased in Saran and held 11 weeks at 5°C.(41°F.) was indistinguishable visually from the freshly made product.

It is recognized that liver sausage in commercial trade channels may not always be kept at temperatures as low as those used in the work cited. Therefore, the purpose of the study reported here was to determine the effect of different temperatures of storage on microbial changes in sausage cased in Saran. Both liver sausage and bologna were studied.

METHODS

The liver sausages were regular commercial products approximately 1.75 inches in diameter by 6 inches long and weighing 10 ounces each. All were obtained from the same production lot at the packing plant. The bologna units were prepared experimentally from a single lot of the mix used in commercial manufacture of this product. The individual units were the same size as the liver sausages. The 200-gauge Saran casing used was obtained from the Dow Chemical Co., Midland, Michigan.

Methods of sampling and culturing the organisms were generally the same as those described previously *(1)*. Because of the small size of the sausages, samples were taken from only two zones, the center and the area adjacent to the casing. Bacteria counts were made by plating appropriate dilutions of each sample in Difco tryptone glucose extract agar for aerobic incubation and in BBL thioglycollate medium (Brewer modified) for anaerobic incubation. All plates were incubated at 30°C.(86°F.) for 5 days. The pH of each sample was measured by the method described by Watson *(3)* for Swiss cheese.

Samples of each kind of sausage were stored at 5°C.(41°F.), 10.5°C.(51°F.), 16°C.(61°F.), and 30°C.(86°F.). The highest temperature was included to represent the most severe conditions that might be found on an unrefrigerated display counter in a retail store.

RESULTS

As might be expected with sausage in Saran casing, the results for the outer and inner zones were practically identical in both products at all

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temperatures of storage. Consequently, only the values for the outer zone are tabulated and discussed.

Liver sausage. Table 1 shows the results for this product. Since all of the sausages represented one production lot, a single unit was chosen at random for determination of the initial values. These are shown in the part of the table devoted to sausage stored at 30°C.(86°F.).

TABLE 1

Effect of temperature of storage on microbial and pH changes in the outer zone of liver sausage

Temperature of storage	Days of storage	Counts per gram in		pH
		TA[b]	TGEA[b]	
30°C.(86°F.)	0	<100	9,300	6.51
	2	4,900	4,900	6.46
	3	1,100,000	1,900,000	6.48
	6	12,000,000	51,000,000	6.72
16°C.(61°F.)	6	<100	6,400	6.58
	10	<100	5,900	6.69
	13	100	3,600	6.53
	19	190,000	350,000	6.49
	24	2,200,000	2,100,000	6.46
	37	3,200,000	4,200,000	6.57
	52	1,400,000	2,200,000	6.56
	59	3,000,000	4,400,000	6.40
	72	3,300,000	2,300,000	6.53
	86	10,000,000	9,300,000	6.58
10.5°C.(51°F.)	8	<100	7,300	.....
	16	<100	8,500	6.57
	23	<100	5,500	6.54
	38	<100	5,700	6.51
	49	<100	7,100	6.60
	58	<100	4,800	6.53
	72	<100	3,800	6.62
	91	<100	3,300	6.54
5°C.(41°F.)	15	<100	11,000	6.51
	51	<100	8,500	6.54
	74	<100	6,600	6.66
	93	<100	5,200	6.54

[b]TA represents thioglycollate agar; TGEA represents tryptone glucose extract agar.

The initial microbial flora consisted mainly of aerobic micrococci. Organisms able to grow anaerobically were very few, less than 100 per gram. The sausage held at 30°C.(86°F.) had a normal appearance after 3 days even though there was abundant growth of about equal numbers of *Micrococcus candidus* and *Micrococcus epidermidis*. After 6 days, however, much gas had been formed and the numbers of organisms were considerably higher than found at the previous sampling period. The predominant organisms here were aerobic spore forming bacilli. These were not identified but at least two types were found, one of which was markedly proteolytic in milk cultures. Presumably the growth of these proteolytic rods caused the rise in pH observed at the last sampling period.

Sausage stored at 16°C.(61°F.) showed no microbial change until the 19th day. Growth continued until the 24th day, after which there was little or no further change in numbers. The predominant organisms during the early stages of growth were *M. candidus* and *M. epidermidis* while at the end of storage the flora was a mixture of these organisms and *Bacillus* spp. The pH showed no change. The sausage appeared normal until the 52nd day, after which it was slightly softer than originally.

As can be seen in Table 1, no change occurred in the flora or pH of sausages stored at 10.5°C.(51°F.) or below. Both lots retained their normal appearance for the duration of the experiment.

TABLE 2

Effect of temperature of storage on microbial and pH changes in the outer zone of bologna

Temperature of storage	Days of storage	Counts per gram in		pH
		TA[c]	TGEA[c]	
30°C.(86°F.)	0	5,900	4,200	6.35
	2	190,000	320,000	6.33
	3	2,000,000	2,300,000	6.37
	4	2,200,000	2,700,000	6.31
	6	2,300,000	2,500,000	6.29
	8	6,100,000	4,600,000	6.36
	9	16,000,000	15,000,000	5.79
	12	14,000,000	13,000,000	5.77
	13	6,100,000	16,000,000	6.11
	15	43,000,000	37,000,000	5.73
	20	14,000,000	12,000,000	5.64
16°C.(61°F.)	3	2,700	7,600	6.35
	8	300	4,700	6.30
	12	200	4,400	6.30
	16	210,000	2,900,000	6.28
	23	2,600,000	2,500,000	6.42
	26	2,800,000	2,700,000	6.28
	32	2,200,000	2,400,000	6.26
	37	6,600,000	6,300.000	5.91
	43	260,000	320,000	6.19
	50	12,000,000	14,000,000	5.99
	57	2,600,000	2,700,000	6.26
10.5°C.(51°F.)	6	5,000	5,900	6.29
	14	<100	4,600	6.26
	29	<100	6,100	6.33
	36	<100	5,300	6.29
	51	<100	6,100	6.24
	62	<100	3,800	6.29
	73	<100	5,500	6.26
	85	360,000	360,000	6.32
	104	1,200,000	1,200,000	6.27
5°C.(41°F.)	14	700	4,900	6.27
	28	200	11,000	6.28
	64	<100	10,000	6.27
	87	<100	8,500	6.33
	106	<100	6,800	6.27

[c] TA represents thioglycollate agar; TGEA represents tryptone glucose extract agar.

Bologna. The results in Table 2 show in general the same trends as those described above for liver sausage. Again a single sausage was chosen at random for determination of the initial counts. The original flora was about the same as that in liver sausage both in numbers and kinds of organisms. In the bologna held at 30°C.(86°F.) the organisms multiplied rapidly during the first 3 days, after which there was little further increase. Gas was evident at 6 days and by 15 days a greenish discoloration appeared. The predominant organism in all samples after 2 days was *M. candidus*. Its extensive growth caused a marked increase in acidity.

Growth in bologna stored at 16°C.(61°F.) was first evident on the 16th day, and after 23 days there was little further increase in numbers. At this temperature the sausage retained its normal appearance for 32 days, after which it was slightly softer than usual. All organisms isolated after growth began at 16 days were *M. candidus*. In spite of the variation between individual sausages, there appeared to be a very slight decrease in pH.

M. candidus was also found after 85 days of storage at 10.5°C.(51°F.) but it did not grow in the sausage held at 5°C.(41°F.) during the 106 days of the experiment. The bologna in both of these lots retained its normal appearance for the entire 15 weeks of storage.

DISCUSSION

These results show that both liver sausage and bologna are very stable products when packaged in Saran and held under fair to good conditions of refrigeration. Even when the products are poorly refrigerated (16°C. or 61°F.), growth of microorganisms is delayed for two weeks or more. As might be expected both types of sausage spoil rapidly when held at or near room temperature.

The organisms initially present and able to grow in liver sausage and bologna comprise only a small fraction of the total microbial flora, most of which is strictly aerobic. The organisms that can grow when provided with a favorable temperature are mainly facultative micrococci that produce acid, in particular *M. candidus*. The growth of *Bacillus* spp. in liver sausage and not in bologna probably can be ascribed to differences in the initial contamination of the two products. There is no evident reason why these organisms should grow in one product and not in the other.

SUMMARY

Both liver sausage and bologna packaged in Saran retained their normal appearance when stored for 13 to 15 weeks at 10.5°C.(51°F.) or lower. When the storage temperature was 16°C.(61°F.), growth was delayed for about 2 weeks, but the numbers of organisms eventually reached a few millions per gram. Bacteria multiplied very rapidly in both products at 30°C.(86°F.).

The predominant organisms that grew in both liver sausage and bologna at the two higher temperatures were micrococci. *Micrococcus candidus* was the only organism isolated from bologna but both *M. candidus* and *M. epidermidis* grew in liver sausage. *Bacillus* spp. also grew in liver sausage at the two higher temperatures, eventually becoming predominant in the sausage held at 30°C.(86°F.).

Acknowledgment

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THE EFFECT OF STORAGE ON INDICATOR ORGANISMS IN SHUCKED OYSTERS AND A COMPARISON OF METHODS FOR THE DETERMINATION OF ENTEROCOCCI[a]

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In those foods whose sanitary quality is evaluated wholly or partly by means of the bacteriological examination, it is important to know whether the organisms employed as indicators of pollution increase or decrease during storage of the food. Oysters are commonly stored at temperatures a little above freezing. The pathogenic intestinal bacteria are unable to multiply under these conditions. However, members of the coliform group may be able to increase at such temperatures. Hunter and Harrison *(4)* stated that "when shucked oysters are stored at temperatures below 10°C. (50°F.) no multiplication of *B. coli* or other lactose fermenting bacteria occurs." Parsons, according to Hunter and Harrison *(4)*, also stated that *B. coli* would not multiply in shell oysters in such a way as to affect the bacterial score. Tonney and White *(9)*, on the other hand, reported that *B. coli* multiplied in shucked oysters stored at 5-8°C.(41-46°F.). McCleskey and Boyd *(6)* found that in crabmeat stored in cracked ice the coliforms tended to increase in numbers while the enterococci and *Escherichia coli* remained practically unchanged. If coliforms are able to multiply under conditions of normal storage of the food, while the pathogens do not, it is obvious that their value as an indicator of pollution is greatly reduced.

Wilson and McCleskey *(10)* found that in shell oysters taken from reefs along the Louisiana coast of the Gulf of Mexico there was a general correlation between the numbers of coliforms and the numbers of *E. coli* and enterococci. Samples containing large numbers of coliforms usually had relatively large numbers of *E. coli* and enterococci, but the actual numbers of the latter were usually not large: half the samples contained no enterococci. They concluded that the enterococci could be employed along with *E. coli* as indicators of pollution in oysters and that they possess the same advantages over the coliform group that has been claimed for *E. coli* by Hunter *(2, 3)*, Perry *(8)*, and Perry and Bayliss *(7)*. The purpose of the present investigation was to provide more information on the behavior of all three indicator organisms in oysters stored under normal conditions of refrigeration.

METHODS

The oysters employed in this investigation were from the Louisiana Gulf coast. Some of the oysters were commercially shucked, and others were procured in the shell and shucked in the laboratory. The oysters shucked in the laboratory were not washed after removal from the shells, and were stored in the shell liquor. All the samples were stored in the refrigerator at 4-6°C.(39-43°F.), and some of the samples were also packed

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TABLE 1

The effect of storage at 4-6°C. (39-43°F.) on coliforms, *E. coli*, and enterococci in laboratory shucked oysters[b]

Sample	Days held	MPN per gram[c]		
		Coliforms	*E. coli*[d]	Enterococci
27	1	33,000	4.90	0.0
	8	.....	0.78	0.0
	13	17,000	7.0	0.0
	20*	4,900,000	2.3	0.0
	29*	35,000,000	2.3	0.2
28	1	79	13.0	0.0
	8	.....	0.0	0.0
	13	33,000	3.30	0.0
	20*	16,000,000	1.70	0.0
	29*	35,000,000	0.45	0.0
29	1	3,300	13.0	4.9
	8	4,900	2.0	11.0
	15	95,000	4.9	4.9
	22*	1,300,000	4.9	0.2
30	1	240	7.9	0.0
	8	400	2.0	0.20
	15	11,000	0.78	0.45
	22*	790,000	0.45	13.0
31	1	22	4.9	1.1
	8	24	3.3	4.9
	15	43	.....	49.0
	21*	2,400	1.1	1,600.0
33	1	49	2.0	0.2
	8	16,000	0.2	4.9
	15	3,500,000	0.68	4.6
	21*	11,000,000	0.0	70.0
45	2	22	0.0	0.0
	8	1,700	0.0	0.0
	14	780	0.0	0.0
	37*	240,000	0.0	0.0
46	2	4.5	0.2	0.0
	12	3,500	0.78	0.45
	31*	24,000	0.0	16,000.0

[b] Oyster bodies were unwashed, and stored in shell liquor.
[c] MPN indicates most probable number of organisms.
[d] *E. coli* determined by the Hajna and Perry *(1)* modification of the Eijkman procedure.
* Represents spoiled as indicated by odor and appearance.

with cracked ice. At the beginning of the storage experiments the laboratory-shucked oysters had been removed from the beds for about 24 hours, and the commercial shucked oysters had been dredged about a week before. The oysters were stored in 1-quart glass jars and thoroughly mixed prior to the removal of portions for examination.

Bacteriological methods for the detection and enumeration of coliforms, *E. coli* and enterococci were those described by Wilson and McCleskey *(10)* except that additional methods for the enterococci were employed in the examination of some of the samples. The azide broth of Rothe as described by Mallman *(5)*, but employed with brom thymol blue indicator, and the "8 F" medium of Hajna and Perry *(1)* were employed in addition to the Winter and Sandholzer *(11, 12)* medium in the presumptive test. The confirmed test was with the Winter and Sandholzer broth-slant method, with

and without penicillin, and "S F" plates. In every case the Gram reaction and morphology were determined.

RESULTS

Twenty-six samples of laboratory-shucked oysters were examined at intervals during storage at 4-6°C.(39-43°F.). The results obtained with 8 typical samples are presented in Table 1. The coliforms increased enormously in most samples; the one exception was a sample containing only 2 coliforms per gram initially. The enterococci remained practically unchanged during the first 8 to 12 days, but increased greatly in some samples after 21 to 31 days. Only 1 sample of the 26 showed a significant decline during storage. *E. coli* decreased in numbers during storage in 24 of the 26 samples, and did not change significantly in the other 2 samples.

The results obtained with the commercially shucked oysters (Table 2) indicate much larger initial numbers of enterococci, and somewhat smaller numbers of *E. coli* than in the laboratory-shucked samples. The reason for this is not obvious, but may be related to the tendency of *E. coli* to die

TABLE 2

The numbers of indicator organisms in shell oysters and in shucked market oysters

	No. of samples	Average MPN per gram of oyster bodies [f]		
		Coliforms	*E. coli* [i]	Enterococci
Shell oysters				
(1)[g]	40	28,850	51.9	22.2
(2)[h]	39	5,485	39.4	2.5
Shucked market oysters	11	170,000	10.3	1270.3

[f] For the meaning of MPN see footnote [c], Table 1.
[g] Including one polluted sample.
[h] Excluding the questionable sample.
[i] *E. coli* as determined by the Hajna and Perry *(1)* method.

TABLE 3

The effect of iced storage at 0 to −1°C.(32-33°F.) on indicator organisms in commercially shucked oysters

Sample No.	Days held [j]	MPN per gram of oyster bodies [k]		
		Coliforms	*E. coli* [i]	Enterococci
48	1	79,000	13.0	490
	10	22,000	2.1	170
	17	79,000	4.5	79
49	1	2,400	4.5	24
	10	6,400	0.78	13
	17	13,000	0.20	4.9
50	1	1,700	1.70	130
	10	7,000	33.0	95
	17	79	2.0	11
51	1	4,900	4.9	350
	10	1,100	1.1	49
	17	23	2.3	24

[j] Days held after purchase.
[k] For the meaning of MPN see footnote [c], Table 1.
[i] *E. coli* as determined by the Hajna and Perry procedure.

TABLE 4

A comparison of methods for the determination of enterococci. Numbers (MPN, most probable number) of enterococci obtained by different methods

Sample No.	Days held	Presumptive media[m]	Winter and Sandholzer (with penicillin)	Winter and Sandholzer (no penicillin)	"S F" plates
48	1	WS	40	27	26
		AD	490	490	490
		SF	24	24	24
	10	WS	170	170	170
		AD	170	170	170
		SF	79	79	79
	17	WS	35	35	3.3
		AD	79	79	79
		SF	13	13	13
49	1	WS	24	24	24
		AD	24	24	24
		SF	1.7	1.7	1.7
	10	WS	11	11	11
		AD	11	11	14
		SF	13	13	13
	17	WS	4.9	4.9	3.3
		AD	2.7	2.7	2.7
		SF	1.8	1.8	1.8
50	1	WS	14	49	14
		AD	130	130	130
		SF	7.9	7.9	7.9
	10	WS	95	95	95
		AD	70	70	70
		SF	24	24	24
	17	WS	7.9	7.9	7.9
		AD	11	11	11
		SF	1.4	1.4	1.4
51	1	WS	49	49	49
		AD	350	350	350
		SF	13	13	13
	10	WS	22	22	22
		AD	49	49	49
		SF	24	24	24
	17	WS	24	24	4.9
		AD	24	24	24
		SF	4.9	4.9	4.9

[m] WS represents Winter and Sandholzer presumptive broth, AD represents azide dextrose broth, SF represents "S F" broth.

during iced storage, or perhaps it may reflect the reduction due to washing the oyster bodies at the time of shucking. In 6 samples of commercial shucked oysters, not shown in tables, there were no significant increases in entercocci and *E. coli* during 8 to 16 days of storage at 4-6°C.(39-43°F.). In most samples the numbers of both organisms decreased, and the coli-

forms increased. When shucked oysters were stored in cracked ice in the refrigerator at 0 to —1°C.(32-33°F.), all three indicator organisms tended to remain constant or to decline in numbers (Table 3).

The three methods employed for the enumeration of enterococci were found to give widely varying results (Tables 4, 5, and 6). The azide dextrose medium of Rothe was found to yield the highest counts in almost all cases, and the average was about three times that obtained with the Winter and Sandholzer medium. The lowest counts were usually obtained with "S F" medium.

TABLE 5

The average MPN (most probable number) obtained by use of various presumptive and confirmatory media in the determination of enterococci

Presumptive medium	Average MPN[a] confirmed in:		
	Winter and Sandholzer (with penicillin)	Winter and Sandholzer (no penicillin)	"S F" plates
Winter and Sandholzer	41.4	43.2	35.9
Azide dextrose broth	117.5	117.5	117.8
"S F" broth	17.3	17.3	17.3

[a]Each figure is the average of 12 samples (see Table 4).

The slant broth confirmatory medium of Winter and Sandholzer was somewhat more effective than "S F" plates when used with the Winter and Sandholzer presumptive medium; the "S F" plates were equally effective when the azide dextrose broth of Rothe was employed as the presumptive medium. The use of penicillin in the Winter and Sandholzer medium did not increase the number of confirmations.

TABLE 6

Comparison of media used in the determination of enterococci

Presumptive medium	No. of positive tubes	Winter and Sandholzer (with penicillin)		Winter and Sandholzer (no penicillin)		"S F" plates	
		No.	%	*No.*	%	*No.*	%
Winter and Sandholzer	125	121	96.8	121	96.8	106	84.8
Azide dextrose broth	147	144	97.9	144	97.9	145	98.6
"S F" broth	100	100	100.0	100	100.0	100	100.0

DISCUSSION

Our results confirm the observation of Tonney and White *(9)* that coliform organisms multiply in shucked oysters during refrigerated storage. The rate of increase is not great during the first few days, however, and the numbers may not change greatly during shipment from the packer to inland cities, provided the temperature is kept low by thorough icing or refrigeration. The comparative constancy in numbers of *E. coli* and enterococci during storage suggests that they are more reliable as indicators of pollution than the coliform group. Large numbers of coliforms and enterococci, with low *E. coli* counts, appear to be indicative of incipient spoilage rather than of pollution.

Of the three media employed for the enumeration of enterococci, azide dextrose broth was the most productive. It was not determined, however, that all of the streptococci grown in this medium were true enterococci. The counts were more consistent when the azide dextrose medium was used than when the other media were employed. The azide dextrose medium apparently permits growth of types, or of small inocula, which are inhibited in the other media as indicated by the higher counts obtained with this medium. The large inocula used in the confirmed test usually assured multiplication on each of the media used for confirmation, therefore the differences in counts were due to the presumptive media, and not to the methods of confirmation.

CONCLUSIONS

Coliform organisms were found to multiply in shucked oysters stored at 4-6°C.(39-43°F.). The increase was slow at first but enormous numbers were present in some samples after 3 to 4 weeks. *E. coli* did not multiply and usually decreased during refrigerated storage of shucked oysters. The enterococci usually remained practically unchanged for about 2 weeks, and often increased after 3 to 4 weeks of storage. *E. coli* appears to be a better indicator of pollution in stored oysters than the coliform group or the enterococci.

The azide dextrose medium of Rothe gave higher counts and more consistent results than the "S F" medium of Hajna and Perry and the medium of Winter and Sandholzer. For the confirmed test, "S F" agar plates or the slant-broth method of Winter and Sandholzer were equally satisfactory if enrichment was in "S F" broth or in the azide dextrose medium. When enrichment was in the Winter and Sandholzer medium the slant-broth method of confirmation was superior. The use of penicillin in the slant-broth medium did not increase the number of confirmations.

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SPORE DESTRUCTION RATE STUDIES ON ORGANISMS OF SIGNIFICANCE IN THE PROCESSING OF CANNED FOODS

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In the development of adequate heat processes for canned foods, consideration must be given to the heat resistance of the significant spoilage organisms. Bigelow *(4)* pointed out that thermal death time data obtained on spores heated at various temperatures resulted in straight line curves when plotted on semi-log paper. Ball *(2)* developed mathematical methods of correlating heat penetration data with thermal death time data to determine the process times necessary for sterilization. He described the thermal death time curve by the following factors: F, which represents the number of minutes required to destroy the organism at 121.11°C.(250°F.), and z, which is the slope of the thermal death time curve (expressed as degrees F. required for the curve to traverse one log cycle of time in minutes).

It is usual practice in evaluating processes for low-acid canned foods to assume a slope of 7.78°C.(18°F.) for the thermal death time curve and express the lethality of a given process as F_0 [the number of minutes required to destroy the organism at 121.11°C.(250°F.) when z is equal to 7.78°C.(18°F.)]. The studies on spore destruction rates reported herein were designed to establish accurate thermal death time curve slope values and to determine the significance of variations in the slope values for the spoilage organisms of significance in the canning of low-acid foods.

Methods commonly used in determining the heat resistance of spores include heating the substrate in sealed glass tubes in oil, water, or steam *(5, 7)* and heating the substrate in thermal death time cans in water or steam *(1)*. The thermal death time tubes may be incubated directly when the substrate is one which will support the growth of the organism being tested, or they may be subcultured into tubes or plates of a suitable medium. The thermal death time cans are customarily incubated directly. With both methods, the usual criterion of death is the failure of the organism to produce growth. The death time is, therefore, bracketed by a maximum survival point and a minimum destruction point.

Townsend, Esty, and Baselt *(14)* reported heat resistance studies on spores of Putrefactive Anaerobe No. 3679 and *Clostridium botulinum* heated in thermal death time tubes of brine from different processed vegetables and in thermal death time cans containing processed and raw corn. These authors suggested that additional work be done with special reference to raw rather than to processed products. Sognesfest, Hays, Wheaton, and Benjamin *(12)* worked with purees prepared from raw vegetables and heated in thermal death time cans in investigating the effect of pH upon process requirements. The z values of the thermal death time curves

TABLE 1

Spore destruction rate of Putrefactive Anaerobe No. 3679

Organism: P. A. No. 3679 (2-24-37) Flask No. 4.
Quantitative medium: Pork agar (shake tubes) incubated 21 days at 27°C.(80.6°F.).
Qualitative medium: Pork broth.

Substrate	Run No.	Temp., °F.	Boiled 5 min., count per ml.	Time in seconds, 212°F. to run temp.	Temp. up, count per ml.	Quantitative		Qualitative		Food tubes		Minutes for 99.99% destruction	pH	
						min. +	min. −	min. +	min. −	min. +	min. −		Initial	Final[b]
Phosphate	5-1	221.0	29,000	18	50,000	275	300	.....	.....	.....	.....	143	7.0	7.0
	6-1	226.4	35,000	20	35,000	90	100	60	70	.....	.....	62	7.0	7.0
	3-7	231.8	31,500	33	30,500	55	60	35	40	.....	.....	34	7.0	7.0
	8-5	239.0	25,500	50	11,000	20	22	.....	.....	.....	.....	20.4	7.0	7.0
	8-6	239.0	28,000	50	11,500	24	.....	18	20	.....	.....	26.2[a]	7.0	7.0
	Low count	239.0	4,000	52	1,300	20	.....	20	.....	.....	.....	31.5[a]	7.0	7.0
Green beans	35	221.0	35,000	14	26,000	175	200	.....	.....	50	75	149	....	....
	36	226.4	50,000	21	40,000	100	.....	.....	.....	30	40	69	....	....
	37-2	231.8	25,000	34	20,000	40	45	.....	.....	.....	5	31.0	....	....
	38	239.0	30,000	55	6,500	12	14	.....	.....	2	4	11.9	....	....
	38-1	239.0	20,000	57	5,500	15	18	.....	.....	.....	3	14.0	6.6	5.6
Green bean puree	23	221.0	11,500	12	7,000	100	120	.....	.....	.....	20	98	5.1	5.0
	24	226.4	24,000	22	5,000	60	70	.....	.....	.....	10	62[a]	5.2	5.1
	25	231.8	20,000	32	5,000	35	40	.....	.....	.....	5	37.1[a]	5.2	5.1
	26	239.0	20,000	51	3,500	20	.....	.....	.....	.....	2	21.4[a]	5.3	5.1
Corn (frozen)	157	221.0	18,000	14	20,000	250	275	.....	.....	225	.....	247[c]	7.2	6.1
	158	226.4	26,000	24	22,000	120	.....	.....	.....	90	.....	124[a, c]	7.2	6.3
	159	231.8	28,000	38	14,000	45	50	.....	.....	45	.....	55[a, c]	7.3	6.6
	160	239.0	38,000	57	6,500	24	27	.....	.....	21	.....	26[c]	7.1	6.5
Corn puree (frozen)	11	221.0	30,000	15	25,000	220	.....	.....	.....	220	.....	210	7.9	7.1
	12	226.4	30,000	29	23,500	60	.....	.....	.....	60	.....	69.8[a]	8.0	7.2
	13	231.8	34,000	28	22,000	20	25	.....	.....	15	20	21.4[a]	8.0	7.4
	14	239.0	25,000	97	3,000	15	18	.....	.....	15	18	16.4[a]	8.0	7.4
Peas, fresh	S-1	226.4	115,000	35	100,000	165	180	.....	.....	.....	.....	113	....	....
	S-2	226.4	95,000	....	51,000	120	135	.....	.....	.....	.....	76.9	....	....
	S-3	233.6	140,000	64	66,000	48	60	.....	.....	.....	.....	42.3	....	....
	S-4	233.6	170,000	55	38,000	42	48	.....	.....	.....	.....	.....	....	....
	S-5	240.8	.....	55	16,000	27	30	.....	.....	.....	.....	25.3	....	....

	S-6	240.8	.....	....	13,500	33	.....	.....	.....	.....	.....	36.1[a]	....	....
	S-7	240.8	115,000	....	5,000	30	33	.....	.....	.....	.....	30.0	....	....
	S-8	240.8	140,000	....	20,000	27	30	.....	.....	.....	.....	.....	....	....
	S-9	240.8	23,000	....	21,000	27	30	.....	.....	.....	.....	19.8	....	....
	S-10	240.8	.....	....	6,000	30	33	.....	.....	.....	.....	28.4	....	....
	S-11	248.0	140,000	90	12,000	13.4	15.4	.....	.....	.....	.....	9.8	....	....
	S-12	248.0	.....	70	6,000	10.6	12.5	.....	.....	.....	.....	11.2[a]	....	....
Peas (frozen)	31	221.0	25,000	10	32,000	360	.....	.....	.....	330	360	319	7.2	5.8
	32	226.4	36,000	24	20,000	165	.....	.....	.....	150	165	142	7.1	5.9
	33	231.8	19,000	34	11,000	72	.....	.....	.....	72	.....	74[a]	7.0	6.0
	34	239.0	24,000	57	3,500	33	.....	.....	.....	33	.....	35.7[a]	7.1	6.1
Pea puree (frozen)	19	221.0	25,000	11	50,000	270	300	.....	.....	210	240	230	7.0	6.2
	20	226.4	20,000	16	25,000	135	150	.....	.....	120	135	117	7.0	6.3
	21	231.8	18,000	29	30,000	64	72	.....	.....	48	56	57.1	7.0	6.4
	22	239.0	24,000	45	5,000	35	40	.....	.....	25	30	33.1	6.9	6.4
Shrimp	E-1	221.0	39,000	13	70,000	225	.....	.....	.....	75	100	175	7.5	7.4
	E-2	226.4	39,000	22	35,000	100	.....	.....	.....	50	60	75	7.4	7.1
	E-3	231.8	40,000	35	34,000	50	.....	.....	.....	30	.....	41.3	7.4	7.2
	E-4	239.0	40,000	52	8,000	21	24	.....	.....	.....	15	24.8[a]	8.2	8.0
Spinach	39	221.0	50,000	15	10,500	200	225	.....	.....	.....	100	181	6.7	5.3
	40	226.4	30,000	17	16,500	110	120	.....	.....	60	70	103	6.9	5.4
	41	231.8	45,000	34	20,000	60	.....	.....	.....	35	40	48.3	6.5	5.6
	42	239.0	45,000	56	10,000	20	.....	.....	.....	16	.....	20.2[a]	7.1	5.8
Spinach puree	27	221.0	20,500	15	30,000	180	210	.....	.....	90	120	169	6.1	5.3
	28	226.4	21,000	17	17,000	105	120	.....	.....	75	90	105.7	6.1	5.5
	29	231.8	19,000	27	12,000	57	64	.....	.....	16	24	53	6.2	5.7
	30	239.0	28,000	41	10,000	36	40	.....	.....	4	8	29	6.3	5.7
Sweet potatoes (7.5% syrup)	145	221.0	20,000	21	25,000	300	330	.....	.....	.....	120	159	....	....
	146	226.4	50,000	31	20,000	100	110	90	100	.....	70	83	6.7	5.8
	147	231.8	29,000	45	8,500	60	.....	50	55	.....	35	43.9	7.1	5.8
	148	239.0	22,000	67	5,500	22	24	12	14	.....	.....	17.0	7.2	6.1
Sweet potatoes (50% syrup)	149	221.0	18,000	24	20,000	300	330	.....	.....	.....	120	209	....	....
	150	226.4	30,000	32	18,000	120	.....	120	.....	.....	80	92	6.8	5.6
	151	231.8	26,000	50	23,000	65	.....	30	35	.....	35	35.5	6.5	5.7
	152	239.0	20,000	87	5,500	28	30	.....	.....	.....	.....	22.8	6.7	5.8

[a] Extrapolated value.
[b] pH at first negative quantitative recovery time, or last positive recovery time when no endpoint was reached.
[c] Minutes for destruction of 99.999%.

TABLE 2

Spore destruction rate of *Cl. botulinum*, type A

Organism: *Cl. botulinum* No. 62-A (3-20-40)
Quantitative medium: Pork agar (shake tubes) incubated 21 days at 27°C.(80.6°F.).
Qualitative medium: Pork broth.

Substrate	Run No.	Temp., °F.	Boiled 5 min., count per ml.	Time in seconds, 212°F. to run temp.	Temp. up, count per ml.	Quantitative		Qualitative		Food tubes		Minutes for 99.999% destruction	pH	
						min. +	min. −	min. +	min. −	min. +	min. −		Initial	Final[4]
Phosphate	55-1	217.4	2,300,000	9	5,300,000	90	101.5	120	130	.....	.....	49.5	7.0	7.0
	56-1	222.8	2,700,000	14	2,000,000	60	65	70	.....	.....	.....	33.0	7.0	7.0
	57-1	228.2	7,000,000	22	820,000	27	30	33	36	.....	.....	17.0	7.0	7.0
	58-2	233.6	3,500,000	33	370,000	12	14	16	18	.....	.....	8.8	7.0	7.0
Phosphate + 0.2% $NaNO_3$	M-A	217.4	5,000,000	8	5,500,000	70	.....	70	.....	.....	.....	38.2	7.0	7.0
	M-B	222.8	4,500,000	14	3,300,000	25	30	45	50	.....	.....	17.7	7.0	7.0
	M-C	228.2	3,000,000	25	1,000,000	21	24	30	33	.....	.....	14.8	7.0	7.0
	M-D	233.6	3,500,000	35	250,000	16	18	18	20	.....	.....	9.9	7.0	7.0
Phosphate + 3.5% NaCl	M-E	217.4	6,000,000	7	1,300,000	40	50	60	70	.....	.....	37.7	7.0	7.0
	M-F	222.8	5,000,000	15	2,200,000	30	35	40	45	.....	.....	20.3	7.0	7.0
	M-G	228.2	5,500,000	24	1,500,000	12	15	21	24	.....	.....	12.3	7.0	7.0
	M-H	233.6	6,000,000	36	170,000	12	14	16	18	.....	.....	10.4	7.0	7.0
Phosphate + 3.5% NaCl + 0.188% $NaNO_3$	M-I	217.4	4,000,000	9	2,200,000	55	60	50	55	.....	.....	33.1	6.6	6.6
	M-J	222.8	5,500,000	19	1,700,000	30	35	45	50	.....	.....	21.5	6.6	6.6
	M-K	228.2	5,000,000	26	1,500,000	21	24	30	.....	.....	.....	14.2	6.6	6.6
	M-L	233.6	6,000,000	38	200,000	10	12	14	16	.....	.....	10.0	6.6	6.6
Green beans	61	217.4	4,500,000	7	4,500,000	100	.....	.....	.....	30	40	47.0	7.3	5.5
	62	222.8	5,500,000	19	2,200,000	35	40	.....	.....	15	20	20.3	7.0	5.8
	63	228.2	1,500,000	24	500,000	18	20	.....	.....	.....	2	12.9	7.2	5.9
	64	233.6	5,000,000	44	450,000	9	10	.....	.....	2	3	7.2	6.9	6.0
Corn, frozen	73	217.4	6,500,000	7	2,500,000	240	.....	.....	.....	240	.....	78	7.1	6.1
	74	222.8	3,000,000	20	1,750,000	78	.....	.....	.....	78	.....	37.2	7.2	6.6
	75	228.2	5,000,000	29	125,000	30	33	.....	.....	21	24	20.7	7.3	6.9
	76	233.6	2,000,000	49	100,000	22	24	.....	.....	18	20	9.4	6.9	6.5
Peas, frozen	69-A	217.4	3,000,000	5	3,000,000	275	300	.....	.....	225	250	72	7.0	5.9
	70-A	222.8	2,000,000	28	300,000	130	.....	.....	.....	130	.....	68	7.0	5.9
	71-A	228.2	2,500,000	31	1,000,000	55	60	.....	.....	55	60	18.2	6.8	6.2
	72-A	233.6	3,000,000	46	200,000	26	28	.....	.....	28	30	21.8	7.2	6.3
Pea puree (frozen)	69	217.4	2,900,000	6	6,000,000	200	225	.....	.....	125	150	62.5	7.1	6.4
	70	222.8	4,000,000	13	3,000,000	80	90	.....	.....	70	80	32.9	7.0	6.5
	71	228.2	2,000,000	23	200,000	30	35	.....	.....	20	25	31.5	6.9	6.6
	72	233.6	500,000	45	100,000	14	16	.....	.....	20	22	13.6	7.2	6.9
Spinach	65	217.4	5,500,000	7	4,300,000	90	100	.....	.....	90	100	62.2	6.7	5.6
	66	222.8	6,500,000	17	2,500,000	50	.....	.....	.....	25	30.5	29.5	6.5	5.9
	67	228.2	4,000,000	28	1,600,000	18	20	.....	.....	14	16	14.7	6.6	6.3
	68	233.6	2,500,000	41	200,000	12	13	.....	.....	6	7	9.6	6.9	6.2

[4] pH at first negative quantitative recovery time, or last positive recovery time when no endpoint was reached.

TABLE 3

Spore destruction rate of *Cl. botulinum*, type B

Organism: *Cl. botulinum* No. 213-B (3-31-38).
Quantitative medium: Pork agar (shake tubes) incubated 21 days at 27°C.(80.6°F.).
Qualitative medium: Pork broth.

Substrate	Run No.	Temp., °F.	Boiled 5 min., count per ml.	Time in seconds, 212°F. to run temp.	Temp. up, count per ml.	Quantitative		Qualitative		Food tubes		Minutes for 99.999% destruction	pH	
						min. +	min. −	min. +	min. −	min. +	min. −		Initial	Final*
Phosphate	51	217.4	3,500,000	12	3,250,000	90	100	90	100	.....	.....	43.2	7.0	7.0
	52	222.8	5,000,000	14	3,100,000	48	54	48	54	.....	.....	31.2	7.0	7.0
	53	228.2	2,200,000	22	3,300,000	30	.....	30	.....	.....	.....	16.0	7.0	7.0
	54	233.6	2,850,000	27	1,350,000	13	14	15	.....	.....	.....	5.6	7.0	7.0
Green beans	77	217.4	2,200,000	10	5,000,000	48	56	.....	.....	.....	8	35	7.1	5.5
	78	222.8	3,400,000	17	3,250,000	40	45	.....	.....	.....	5	18	7.4	5.7
	79	228.2	2,900,000	29	2,100,000	20	22	.....	.....	2	4	10.5	7.0	5.8
	80	233.6	2,000,000	40	350,000	14	15	.....	.....	1	2	6.6	6.9	6.0
Peas, frozen	82	222.8	1,850,000	22	2,200,000	90	100	.....	.....	100	110	.....	6.9	6.1
	83	228.2	3,000,000	34	1,050,000	40	45	.....	.....	40	45	20.2	7.1	6.2
	84	233.6	2,300,000	42	500,000	21	24	.....	.....	15	18	.....	6.8	5.9

*pH at first negative quantitative recovery time, or last positive recovery time when no endpoint was reached.

TABLE 4

Spore destruction rate of Thermophilic Anaerobe No. 3814

Organism: T.A. No. 3814 (3-5-36) 1st centrifuge.

Quantitative medium: Norit-treated tryptone agar shake tubes incubated 7 days at 55°C.(131°F.).

Substrate	Run No.	Temp., °F.	Boiled 5 min., count per ml.	Time in seconds, 212°F. to run temp.	Temp. up, count per ml.	Quantitative		Qualitative		Food tubes		Minutes for 99.99% destruction	pH	
						min. +	min. −	min. +	min. −	min. +	min. −		Initial	Final[1]
Phosphate	85-2	230	70,000	18	62,000	180	.....	.....	.....	.....	.....	102	7.0	7.0
	86-3	233.6	66,500	25	46,000	80	88	.....	.....	.....	.....	48.9	7.0	7.0
	87-3	239	66,000	34	40,000	24	27	.....	.....	.....	.....	16.8	7.0	7.0
	88-3	242.6	63,000	66	13,000	12	14	.....	.....	.....	.....	9.6	7.0	7.0
Green beans	91	230	39,000	25	37,000	175	200	.....	.....	175	200	133.3	6.8	5.3
	92-1	233.6	37,000	29	23,000	110	120	.....	.....	110	120	82.5	7.9	5.2
	93-1	239	41,500	46	22,500	36	.....	.....	.....	36	.....	25.7	7.7	5.5
	94-1	242.6	76,000	60	18,100	16	.....	.....	.....	5	.....	13.0	7.8	6.0
Corn, frozen	99	230	54,000	60	24,000	210	240	.....	.....	180	240	188	7.5	6.0
	100	233.6	40,000	37	31,000	120	135	.....	.....	105	120	75	7.1	6.1
	101	239	40,000	51	17,500	35	40	.....	.....	35	40	25.0	7.2	6.2
	102	242.6	38,000	65	10,500	12	14	.....	.....	16	18	12.2	7.5	6.8
Peas, frozen	95	230	29,000	39	17,000	300	.....	.....	.....	225	250	189	7.3	5.6
	96	233.6	31,000	41	23,300	135	150	.....	.....	150	165	89.5	7.0	5.8
	97	239	35,000	91	16,000	44	48	.....	.....	44	48	31.4	7.0	6.1
	98	242.6	46,000	87	14,000	16	18	.....	.....	16	18	13.5	7.2	6.5
Spinach	103	230	33,000	50	24,000	175	200	.....	.....	150	175	133	7.1	5.4
	104	233.6	42,000	38	25,500	100	.....	.....	.....	100	.....	79.3	7.0	5.7
	105	239	39,000	44	18,600	30	35	.....	.....	30	35	25.6	7.1	6.3
	106	242.6	33,000	55	9,500	16	18	.....	.....	16	18	14.8	7.1	6.1

[1] pH at first negative quantitative recovery time, or last positive recovery time when no endpoint was reached.

reported in both of the above papers were determined by drawing the curve above all survival points and below as many destruction points as possible.

Rahn *(19)* reviewed the experimental evidence supporting the belief that the order of bacterial death by heat is logarithmic. The preponderance of evidence indicated that the curve of the number of organisms surviving plotted against the length of time of the heat treatment should be a straight line on semi-log paper. Establishment of the slope of this survivor curve would permit the selection of a definite time for the desired degree of spore destruction and, consequently, simplify the establishment of the slope of the thermal death time curve.

Ball *(3)* referred to a thermal death time curve whose slope was determined by use of survivor curves as a "Phantom Thermal Death Time Curve" and pointed out that the choice of a destruction time depends upon the nature of the organism, the nature of the food under consideration, and the number of organisms originally present in the food. Stumbo *(13)* suggested that the symbols originally used by Ball be re-defined as follows: U to represent the number of minutes required at a given temperature to reduce the number of organisms to any desired level, and U to equal $Z(\log a - \log b)$ where Z represents the slope of the survivor curve for the organism in question; Z being expressed as the number of minutes required for the survivor curve to traverse one logarithmic cycle of number of organisms; a represents the total number of organisms in the given quantity of food; and b represents the number of organisms to remain viable in the given quantity of food at the end of the heating period.

In a series of papers on the heat resistance of thermophilic bacteria, Gillespy *(8)* used D as an abbreviation of decimal reduction time to represent the slope of the survivor curve. Since some confusion might arise from the use of z to represent the slope of the thermal death time curve and Z to represent the slope of the survivor curve, it has been suggested by Schmidt *(11)* that the symbol D used by Gillespy be adopted.

In the studies reported herein the symbols F and z are used to describe the thermal death time curve and D is used to represent the slope of the survivor curve.

EXPERIMENTAL

Preparation of Spore Suspensions

Sufficient quantities of each spore suspension were prepared for use in all tests. The spore crop identification and preparation methods were as follows:

Putrefactive Anaerobe (P.A.) No. 3679 (2-24-37) Flask No. 4. An actively growing culture of No. 3679 was inoculated into three tubes of pork broth medium which were stratified with pork agar medium and incubated at 37°C.(98.6°F.) for one day. Two-ml. quantities of these tubes were inoculated into 6 tubes of pork broth medium, containing freshly pickled iron strips, which were incubated at 37°C. for one day. The contents of 3 tubes were transferred into each of two flasks containing 50 ml. of pork broth medium and 5 iron strips. The flasks were incubated at 37°C. for 2 days, and their contents were then transferred into a single flask containing 5 liters of pork broth medium and 15 bent iron strips. This flask was incubated at 37°C. for one week followed by 30°C.(86°F.) incubation for two weeks. The broth was then strained through a sterile cheesecloth-cotton pad filter and centrifuged. The spores were taken up in sterile tap water and placed under refrigeration in a bottle containing a small amount of sterile sand.

TABLE 5

Spore destruction rate of Flat Sour Organism No. 1518

Organism: F.S. No. 1518 (3-8-35).
Quantitative medium: B.C.P. tryptone dextrose agar (plates) incubated 2 days at 55°C. (131°F.).
Qualitative medium: B.C.P. tryptone dextrose broth.

Substrate	Run No.	Temp., °F.	Boiled 5 min., count per ml.	Time, 212°F. to run temp.	Temp. up, count per ml.	Quantitative		Qualitative		Food tubes		Minutes for 99.99% destruction	pH	
						min. +	min. −	min. +	min. −	min. +	min. −		Initial	Final[a]
Phosphate	107	230.0	21,000	41 sec.	21,500	300	.....	.....	.....	.....	.....	262	7.0	7.0
	107-1	230.0	17,000	41 sec.	23,000	300	.....	.....	.....	.....	.....	255	7.0	7.0
	108	237.2	25,500	82 sec.	22,700	120	.....	110	120	.....	.....	119	7.0	7.0
	108-1	237.2	19,000	99 sec.	17,000	130	.....	.....	.....	.....	.....	106	7.0	7.0
	109	242.6	16,300	3 min., 35 sec.	15,700	55	60	.....	.....	.....	.....	44.5	7.0	7.0
	109-1	242.6	16,800	2 min., 20 sec.	17,700	55	60	.....	.....	.....	.....	41.4	7.0	7.0
	109-2	242.6	19,000	96 sec.	19,800	65	70	.....	.....	.....	.....	40.7	7.0	7.0
	Special high	242.6	156,000	1 min., 6 sec.	95,000	75	80	.....	.....	.....	.....	60.7	7.0	7.0
	Special low	242.6	650	1 min., 5 sec.	950	51	.....	.....	.....	.....	.....	68.5[b]	7.0	7.0
	110	248.0	22,800	2 min., 36 sec.	20,000	22	27	.....	.....	.....	.....	19.3	7.0	7.0
	110-1	248.0	21,700	2 min., 54 sec.	18,800	32	.....	.....	.....	.....	.....	29.6	7.0	7.0
	110-2	248.0	20,100	3 min., 5 sec.	15,300	25	27	.....	.....	.....	.....	19.3	7.0	7.0
	110-3	248.0	17,100	3 min., 14 sec.	11,300	30	32	.....	.....	.....	.....	21.5	7.0	7.0
Asparagus	125	230.0	13,600	38 sec.	14,600	250	275	200	225	.....	.....	213	6.1	5.3
	126	237.2	18,000	60 sec.	15,400	110	120	100	110	.....	.....	100	6.3	5.3
	127	242.6	21,600	73 sec.	15,400	50	55	50	55	.....	.....	47.1	6.2	5.4
	128	248.0	18,000	1 min., 37 sec.	13,500	30	33	30	33	.....	.....	26.8	6.2	5.4
Green beans	113	230.0	19,300	51 sec.	16,100	275	.....	275	.....	.....	.....	273	6.8	5.2
	114	237.2	18,500	67 sec.	12,900	110	120	.....	.....	.....	.....	97	7.3	5.2
	115	242.6	15,200	2 min.	10,700	50	.....	50	.....	.....	.....	50.2	6.6	5.2
	115-1	242.6	14,000	1 min., 48 sec.	8,700	55	.....	55	.....	.....	.....	54.3	7.3	5.4
	116-1	248.0	17,400	2 min., 57 sec.	10,000	30	.....	.....	.....	.....	.....	25.8[b]	6.4	5.4
Corn, frozen	153	230.0	21,800	29 sec.	14,600	300	330	.....	.....	270	300	251	7.5	5.9
	154	237.2	15,600	51 sec.	12,400	132	.....	.....	.....	120	.....	121	7.0	6.1
	155	242.6	16,000	67 sec.	7,400	60	.....	.....	.....	.....	.....	64.4[b]	6.9	6.3
	156	248.0	16,300	88 sec.	6,700	36	.....	.....	.....	32	.....	36.6[b]	7.0	6.4
Corn puree (frozen)	169	230.0	24,300	49 sec.	22,000	360	.....	.....	.....	360	.....	366[b]	8.2	6.8
	170	237.2	12,800	78 sec.	17,400	180	.....	.....	.....	165	180	161	8.2	6.8
	171	242.6	15,200	2 min., 5 sec.	17,100	80	88	.....	.....	80	88	75.7	8.0	6.8
	172	248.0	14,500	3 min., 23 sec.	14,700	40	44	.....	.....	40	44	35.9	7.8	7.0

Peas, fresh	117	230.0	34,000	40 sec.	28,100	300	.....	.....	.....	.....	.....	337[b]	6.6	5.4
	118	237.2	24,200	52 sec.	21,700	130	140	110	120	.....	.....	112.8	6.5	5.5
	119	242.6	23,100	2 min., 10 sec.	20,600	65	.....	65	.....	.....	.....	64.2	6.8	5.5
	120	248.0	18,700	2 min., 25 sec.	16,100	30	.....	27	30	.....	.....	27.5	6.3	5.5
Peas, frozen	117-1	230.0	19,200	37 sec.	20,500	360	.....	.....	.....	360	.....	296	7.2	5.5
	118-1	237.2	20,900	55 sec.	18,500	180	.....	.....	.....	180	.....	150	7.2	5.7
	119-1	242.6	21,300	75 sec.	20,400	80	88	.....	.....	80	88	72.3	7.4	5.7
	120-1	248.0	23,000	1 min., 44 sec.	23,500	36	.....	.....	.....	.....	.....	37.5[b]	6.9	6.1
Pea puree (frozen)	165	230.0	12,800	23 sec.	16,000	270	300	.....	.....	180	210	264	7.0	6.1
	166	237.2	19,000	47 sec.	20,600	100	120	.....	.....	.....	100	103	7.1	6.2
	167	242.6	20,200	62 sec.	16,800	80	90	.....	.....	.....	50	62.4	7.1	6.1
	168	248.0	17,400	76 sec.	18,000	35	40	.....	.....	.....	30	31.9	6.9	6.2
Pumpkin puree	161	230.0	19,800	33 sec.	16,300	140	160	.....	.....	.....	0	114	5.3	5.0
	162	237.2	18,500	57 sec.	17,500	70	80	.....	.....	.....	0	71.2	5.5	5.1
	163	242.6	13,200	84 sec.	14,200	40	45	.....	.....	.....	0	40.3	5.5	5.2
	164	248.0	17,300	1 min., 46 sec.	19,900	21	24	.....	.....	.....	3	18.9	5.4	5.2
Shrimp	121	230.0	16,000	37 sec.	12,800	325	.....	.....	.....	.....	.....	394[b]	7.2	7.4
	122	237.2	21,350	53 sec.	18,000	130	.....	.....	.....	.....	.....	115	7.2	7.2
	123	242.6	28,500	67 sec.	21,500	80	.....	70	75	.....	.....	57.7	7.0	7.0
	124	248.0	20,300	1 min., 41 sec.	14,100	39	.....	36	39	.....	.....	27.3	7.4	7.3
Spinach	141	230.0	19,200	38 sec.	18,300	240	270	.....	.....	.....	0	216	6.7	5.4
	142	237.2	17,000	30 sec.	13,600	105	120	.....	.....	.....	30	107	6.6	5.7
	143	242.6	20,200	66 sec.	17,200	70	.....	65	70	.....	30	61.4	6.7	5.6
	144	248.0	21,200	2 min.	18,900	33	36	27	30	.....	.....	28.5	6.6	5.7
Sweet potatoes (7.5% syrup)	133	230.0	17,700	35 sec.	18,600	225	250	225	250	.....	.....	213	6.4	5.4
	134	237.2	18,800	65 sec.	16,500	110	120	80	90	.....	.....	88	6.3	5.4
	135	242.6	12,900	84 sec.	14,100	60	65	60	65	.....	.....	52.2	6.3	5.6
	136	248.0	18,800	1 min., 50 sec.	17,800	30	33	30	33	.....	.....	27.8	6.7	5.8
	136-1	248.0	16,200	1 min., 45 sec.	17,100	30	33	30	33	.....	.....	27.5	6.3	5.8
Sweet potatoes (30% syrup)	137	230.0	12,100	43 sec.	10,400	225	250	200	225	.....	.....	217	7.0	5.3
	138	237.2	12,500	68 sec.	15,100	110	120	110	120	.....	.....	105	6.3	5.3
	139	242.6	10,600	97 sec.	15,700	60	65	60	65	.....	.....	59.3	6.4	5.5
	140	248.0	16,500	2 min., 1 sec.	17,600	33	.....	30	33	.....	.....	30.4	6.4	5.7
Sweet potatoes (50% syrup)	140-A	248.0	11,800	2 min., 15 sec.	18,500	36	39	36	39	.....	.....	33	6.5	5.5

[a] pH at first negative quantitative recovery time, or last positive recovery time when no endppoint was reached.
[b] Extrapolated value.

Thermophilic Anaerobe (T.A.) No. 3814 (3-5-36) 1st Centrifuge. An actively growing culture was prepared by transferring portions of a culture of No. 3814 stored in liver broth with added calcium carbonate into tubes of liver broth which were then stratified with agar and incubated at 55°C.(131°F.). Unstratified tubes of liver broth (with meat and iron strips) were inoculated with the active culture and incubated at 55°C. for one day. The contents of two tubes were then transferred into a flask, containing 100 ml. of liver broth (with meat and bent iron strips), which was incubated at 55°C. for one day. The contents of this flask were transferred into a flask containing 600 ml. of liver broth (with meat and bent iron strips). After incubation at 55°C.(131°F.) for one day, the contents were transferred into a flask containing 6 liters of liver broth (with meat and bent iron strips) which was incubated at 55°C. for two days, followed by 42°C.(107.6°F.) incubation for 10 days. The culture was allowed to cool and was then strained through a sterile cheesecloth-cotton pad filter and centrifuged. The spores were taken up in sterile tap water and placed under refrigeration in a bottle containing a small amount of sterile sand.

Flat Sour (F.S.) No. 1518 (3-8-35). Several slants of Difco nutrient agar were inoculated with a culture of No. 1518 and incubated at 55°C.(131°F.) for two days. Each slant was then washed with 10 ml. of sterile water which was then used to inoculate Kolle flasks containing Difco nutrient agar. The Kolle flasks were incubated at 55°C. for two days. The spore crop was washed down with sterile distilled water and was aseptically filtered through sterile cheesecloth prior to storing under refrigeration in sealed test tubes.

Clostridium botulinum No. 62-A (3-20-40) and No. 213-B (3-31-38). The original cultures were obtained from Dr. K. F. Meyer's collection at the Hooper Foundation for Medical Research, University of California. Actively growing cultures in Difco beef heart infusion were inoculated into flasks of freshly exhausted casein digest medium. The flasks were stratified with sterile vaseline and incubated at 30°C.(86°F.) for 3 weeks. The spore crop was concentrated by centrifuging and was re-suspended in a small quantity of the mother liquor and shaken with glass beads. After being filtered through a double layer of sterile cheesecloth, the suspension was heated for 5 minutes in boiling water and stored in the mother liquor under refrigeration.

For the preparation of the bacteriological media used in this work see the Appendix.

Preparation of Substrates

Reference substrate. Standard M/15 phosphate buffer solution (pH 7.0) was used for reference substrate.

Food substrates, non-homogeneous. Asparagus. A weight of 260 g. of freshly washed raw asparagus tips (2½ to 3 inches in length) was blanched for 3 minutes in water at 82.2°C.(180°F.). The blanched tips were placed upright in the "tank" basket and covered with 550 ml. of inoculated 3% salt brine.

Beans, green. A weight of 250 g. of fresh, cut green beans was blanched for 1½ minutes in boiling water. The blanched beans were placed in the "tank" basket and covered with 500 ml. of inoculated 2% salt brine.

Corn, yellow (frozen). One package (360 g.) of Birdseye Golden Bantam Frozen Corn was thawed in cold water. The thawed corn was placed in the "tank" basket and covered with 500 ml. of inoculated brine containing 1% salt and 2% sugar.

Peas (fresh). For tests using the flat sour organism, a weight of 300 g. of freshly hulled peas was blanched for 3 minutes in boiling water. The blanched peas were placed in the "tank" basket and covered with 500 ml. of inoculated brine containing 1.8% salt and 2.2% sugar.

The tests on Putrefactive Anaerobe No. 3679 were conducted at a commercial canning plant. Perfection and Profusion sweet peas (sieve sizes 4 to 6) were used with a brine containing 2.2% salt and 4.8% sugar.

Peas (frozen). One package (360 g.) of Birdseye Frozen Peas were thawed in cold water. The thawed peas were placed in the "tank" basket and covered with 500 ml. of inoculated brine containing 1.8% salt and 2.2% sugar.

Shrimp. A weight of 390 g. of freshly peeled shrimp was blanched for 5 minutes in a boiling 12 to 12.5% salt brine. The blanched shrimp, weighing approximately 280 g., were placed in the "tank" basket and covered with 500 ml. of inoculated 2% salt brine.

Spinach. A weight of 300 g. of fresh, washed spinach was blanched for 3 minutes in water at 93.3°C.(200°F.). The blanched spinach was placed in the "tank" basket and covered with 500 ml. of inoculated 2.5% salt brine.

Sweet potatoes. Nancy Hall sweet potatoes were peeled and sliced by hand. A weight of 260 to 290 g. of potatoes was placed in the "tank" basket and covered with 500 ml. of inoculated sugar syrup. Syrup concentrations of 7.5%, 30%, and 50% were used.

TABLE 6

Destruction curve characteristics for Putrefactive Anaerobe No. 3679

Organism: P.A. No. 3679 (2-24-37); flask No. 4; 27°C.(80.6°F.) incubation.

Substrate	Quantitative in pork agar shake tubes		Direct incubation in food		Calculated F and z value (by method of least squares) for				Run No.
					99.99% destruction		99.999% destruction		
	F	z	F	z	F	z	F	z	
Phosphate (pH 7.0)	.....	.....	.....	.....	5.8	21.4	.....	.....	5-1, 6-1, 3-7, 8-5
	6.1	17.2	.....	.....	7.3	23.8	.....	.....	5-1, 6-1, 3-7, 8-5, 8-6
Green beans	4.1	16.9	1.4	16.9	2.8	16.9	.....	.....	35, 36, 37-2, 38, 38-1
Green bean puree	8.8	26.6	<0.5	<18	8.0	26.6	.....	.....	23 to 26
Corn (frozen)	6.8	18.1	>5.7	>18.1	.....	.....	6.0	18.1	157 to 160
Corn puree (frozen)	3.4	15.4	3.4	15.4	2.4	15.4	.....	.....	11 to 14
Peas (fresh)	13	24	.....	.....	10.2	24	.....	.....	S-1 to S-3, S-5 to S-7, S-9 to S-12
Peas (frozen)	>10	>18.8	8.3	18.8	8.3	18.8	.....	.....	31 to 34
Pea puree (frozen)	12	21.2	9.4	21.2	9.4	21.2	.....	.....	19 to 22
Shrimp	11	21.4	4.8	21.4	6.7	21.4	.....	.....	E-1 to E-4
Spinach	6.5	18.3	6.0	22.6	4.9	18.3	.....	.....	39 to 42
Spinach puree	12	22.9	2.5	15.4	9.3	22.9	.....	.....	27 to 30
Sweet potatoes (7.5% syrup)	6.0	18.7	<3.7	<18.7	4.4	18.7	.....	.....	145 to 148
Sweet potatoes (50% syrup)	7.2	17.9	<3.8	<17.9	4.6	17.9	.....	.....	149 to 152

Food substrates, homogeneous. Beans, green, puree. Commercially prepared, unprocessed strained green beans were obtained and held under dry ice refrigeration. After thawing, 450 ml. of puree were mixed with 250 ml. of water, inoculated, and placed in the "tank."

Corn, yellow, puree (frozen). One package (360 g.) of Birdseye Golden Bantam Frozen Corn was thawed in cold water and blended in a Waring Blendor at 8,000 r.p.m. for one minute with 200 ml. of water. The blended corn was passed through an 0.04-inch screen on a Sepro-sieve, made up to a final volume of 700 ml. by adding approximately 200 ml. of water, inoculated, and placed in the "tank."

Pea puree (frozen). Two packages (720 g.) of Birdseye Frozen Peas were thawed in cold water. The thawed peas were passed through an 0.04-inch screen on a Sepro-

TABLE 7

Destruction curve characteristics for *Cl. botulinum*, type A

Organism: *Cl. botulinum* No. 62-A (3-20-40); 27°C.(80.6°F.) incubation.

Substrate	Quantitative in pork agar shake tubes		Qualitative		Direct incubation in food		Calculated F and z value (by method of least squares) for				Run No.
							99.99% destruction		99.999% destruction		
	F	z	F	z	F	z	F	z	F	z	
Phosphate (pH 7.0)	3.1	20.9	3.6	20.9	.....	.....	1.43	22.6	1.54	20.9	55-1, 56-1, 57-1, 58-2
Phosphate + 0.2% $NaNO_3$	.....	.....	5.7	29.3	.....	.....	.....	.....	2.68	29.3	M-A to M-D
Phosphate + 3.5% NaCl	3.6	28.4	4.7	28.4	.....	.....	.....	.....	2.58	28.4	M-E to M-H
Phosphate + { 0.2% $NaNO_3$ / 3.5% NaCl }	4.8	30.2	6.1	30.2	.....	.....	.....	.....	2.79	30.2	M-I to M-L
Green beans	0.84	15.5	.....	.....	0.21	14.2	.....	.....	1.08	20.3	61 to 64
Frozen corn	2.8	17.7	.....	.....	1.4	14.2	.....	.....	1.12	17.7	73 to 76
Frozen peas	2.4	15.5	.....	.....	2.4	15.5	.....	.....	.....	.....	69-A to 72-A
Frozen pea puree	1.0	14	.....	.....	3.1	19.8	.....	.....	.....	.....	69 to 72
Spinach	2.1	19.4	.....	.....	0.52	14.4	.....	.....	1.22	19.4	65 to 68

sieve, made up to a final volume of 700 ml. by adding 200 to 250 ml. of water, inoculated, and placed in the "tank."

Pumpkin puree. Yellow squash was broken into pieces, the stem and seeds removed, and steamed for 25 minutes under 10 p.s.i.g. pressure. The juice was saved for dilution of the puree. The steamed squash was chopped and passed through an 0.04-inch screen on a Sepro-sieve. The resultant puree was diluted with squash juice and water to the desired consistency.

Spinach puree. Commercially prepared, unprocessed strained spinach was obtained and held under dry ice refrigeration. After thawing, 450 ml. of puree was mixed with 250 ml. of water, inoculated, and placed in the "tank."

LIST OF SUBSTRATES AND ORGANISMS HEATED IN EACH SUBSTRATE

Reference Substrate	Organisms
Neutral phosphate buffer	F.S., T.A., P.A., *Clostridium botulinum* types A and B
Food Substrates	
Non-homogeneous	
Asparagus	F.S.
Beans, green	F.S., T.A., P.A., *Clostridium botulinum* types A and B
Corn, yellow (frozen)	F.S., T.A., P.A., *Clostridium botulinum* type A
Peas (fresh)	F.S., P.A.
Peas (frozen)	F.S., T.A., P.A., *Clostridium botulinum* types A and B
Shrimp	F.S., P.A.
Spinach	F.S., T.A., P.A., *Clostridium botulinum* type A
Sweet potatoes (7.5% syrup)	F.S., P.A.
Sweet potatoes (30% syrup)	F.S.
Sweet potatoes (50% syrup)	P.A.
Homogeneous	
Beans, green, puree	P.A.
Corn, yellow, puree (frozen)	P.A.
Pea puree (frozen)	P.A., *Clostridium botulinum* type A
Pumpkin puree	F.S.
Spinach puree	P.A.

SPORE DESTRUCTION RATE TESTS

The heating tests for the determination of spore destruction rates were made in the "tank" apparatus developed by Williams, Merrill, and Cameron *(15)*. The "tank" consists of a cylindrical chamber, having a capacity of approximately 900 ml., which is heated by a steam jacket. The contents of the chamber are vigorously agitated throughout the test by two four-bladed propellers mounted on a single shaft so as to set up opposing currents and provide rapid heat penetration and uniform spore dispersion. The contents are held at the desired temperature within a normal variation of $\pm 0.1°C.$ ($0.2°F.$). Homogeneous substrates are inoculated and poured directly into the cylindrical chamber. Non-homogeneous substrates are placed in a stainless steel mesh basket, specially designed to fit around the agitator shaft and rest upon the top of the sampling valve supports, and are then covered with the inoculated brine or syrup. Samples are drawn aseptically from the sampling tubes at the desired time intervals.

In each test, spore counts were made on the following samples:

OA Sample: A sample of the substrate, inoculated and mixed with the appropriate spore suspensions, was placed in a sterile test tube and heated for 5 minutes in boiling water.

O Sample: A sample was drawn from the "tank" when the contents reached the holding temperature for the particular test. This sample was used for the original count in determining the percentage of survivors at the various time intervals.

Interval Samples: Samples were drawn from the "tank" at various time intervals after the holding temperature was reached. A portion of each sample was used to determine the pH.

METHODS OF DETERMINING SPORE COUNTS

Many media and spore counting techniques were investigated to determine those giving consistent, maximum spore recoveries. The following methods were adopted for use, with a minimum of three tubes or plates being counted for each dilution:

Putrefactive anaerobe and Clostridium botulinum. Appropriate sample dilutions were cultured in pork agar medium in the deep shake tubes described by Miller, Garrett, and Prickett *(9)*. Prior to tubing, the pork agar medium was sterilized in liter

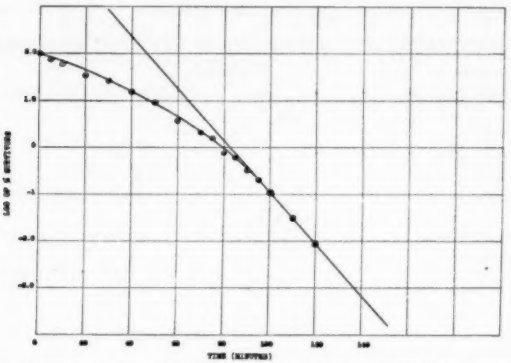

FIGURE 1. Survivor curve of Flat Sour No. 1518 in phosphate buffer (pH 7.0). Heated at 114°C.(237.2°F.). Run No. 108.

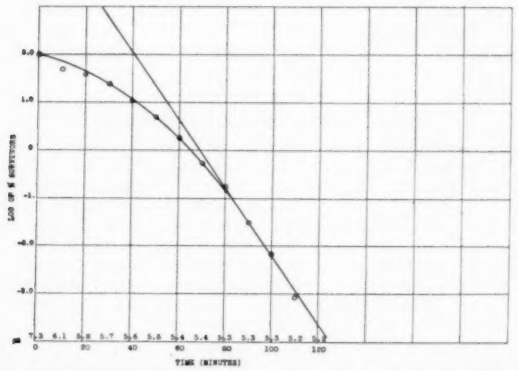

FIGURE 2. Survivor curve of Flat Sour No. 1518 in green beans. Heated at 114°C. (237.2°F.). Run No. 114.

flasks, held overnight at 55°C.(131°F.), and decanted to remove sediment. The inoculated pork agar in the tubes were solidified by immersing the tubes in ice water and was then covered with approximately one-half inch of 2% agar and a layer of sterile mineral oil. The tubes were incubated at 27°C.(81°F.) for a minimum period of 21 days, and colonies were counted at frequent intervals during the incubation period.

Thermophilic anaerobe. Appropriate sample dilutions were cultured in Norit-treated tryptone agar in deep shake tubes. The inoculated agar in the tubes was solidified by

immersing the tubes in ice water and was then covered with approximately one-half inch of 2% agar and a layer of sterile mineral oil. The tubes were incubated at 55°C. (131°F.) for 7 days, and colonies were counted at frequent intervals during the incubation period.

Flat sour. Appropriate sample dilutions were cultured in Petri plates in Difco bactodextrose tryptone agar. The inoculated plates were incubated at 55°C.(131°F.) for 2 days.

Additional Spore Recovery Tests

The duration and temperature of incubation for these tests was the same as that used for the respective quantitative recovery tests.

Tubes of pork or tryptone broth, for anaerobes, or tryptone broth with added Bromcresol purple, for the flat sour organism, were used to give a qualitative check on the thermal death time in some tests.

In a number of tests, 1 x 8 inch test tubes of the food substrate were prepared, sterilized, and inoculated with portions of the interval samples. These food tubes provided a measure of the inhibition of growth of the organisms by the food itself.

TABLE 8

Destruction curve characteristics for *Cl. botulinum*, type B
Organism: *Cl. botulinum* No. 213-B (3-31-38); 27°C.(80.6°F.) incubation.

Substrate	Quantitative in pork agar shake tubes		Qualitative		Direct incubation in food		Calculated F and z value (by method of least squares) for 99.999% destruction		Run No.
	F	z	F	z	F	z	F	z	
Phosphate (pH 7.0)	2.0	17.9	2.0	17.9	.....	.....	0.79	17.9	51 to 54
Green beans	2.5	21.7	.....	.....	0.224	21.7	1.09	21.7	77 to 80
Frozen peas	2.5	17.2	.....	.....	1.1	13.8	.....	.....	81 to 84

Methods of Calculations

The pertinent data on each test and the indicated destruction times are presented in Tables 1 to 5. The calculated F and z values based on percentage destruction are presented in Tables 6 to 10, together with F and z values for the thermal death time indicated by quantitative, qualitative, and food tube endpoints. Table 11 lists the z values obtained for the spores of the several organisms.

The method used in calculating the z values was as follows:

1. For each test, the log of the percentage of survivors per ml. (using the zero sample spore count as the original spore concentration) was plotted against time on coordinate paper.
2. Since the survivor curve could not, in all tests, be expressed as a single straight line passing through the origin, the best smooth curve was drawn by visual inspection.
3. The time for 99.99% destruction (0.01% survival) or the time for 99.999% destruction (0.001% survival) was taken from the survivor curve.
4. By the method of least squares, the slope and intercept of the regression line for the percentage destruction times, plotted against temperature on semi-log paper, was calculated. The formula used was in the form stated by Worthing and Geffner *(16)*: $y = a + b\,x$; where: y is the log time, a the log F, b the reciprocal of z, and x is (250°F. — temp. of test); and:

$$a = \frac{\Sigma x^2 \Sigma y - \Sigma x \Sigma x y}{n \Sigma x^2 - (\Sigma x)^2}; \text{ while } b = \frac{n \Sigma x y - \Sigma x \Sigma y}{n \Sigma x^2 - (\Sigma x)^2}$$

In applying the calculated z value to quantitative, qualitative, and food tube endpoints, the thermal death time curve was drawn so as to fall above all survival points and below as many destruction points as possible.

DISCUSSION

The experimental program was initiated during 1938 with the hope that the survivor curve might be expressed as a single straight line passing through the origin when the log of the percentage of survivors was plotted against time. The "Phantom Thermal Death Time Curve" estab-

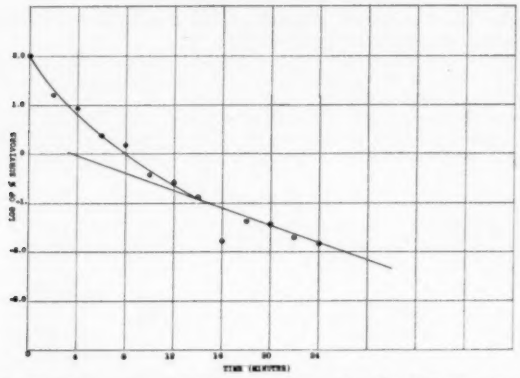

FIGURE 3. Survivor curve of Putrefactive Anaerobe No. 3679 in phosphate buffer (pH 7.0). Heated at 115°C.(239°F.). Run No. 8-6.

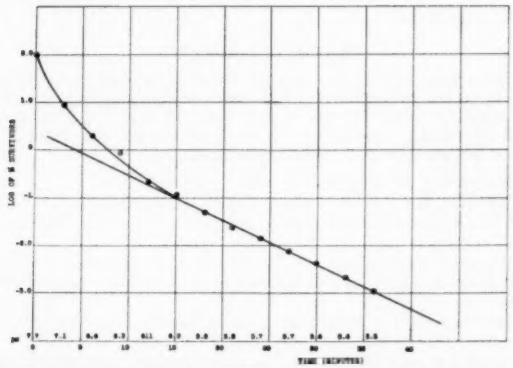

FIGURE 4. Survivor curve of Thermophilic Anaerobe No. 3814 in green beans. Heated at 115°C.(239°F.). Run No. 93-1.

lished by the *D* values (slopes) of the survivor curves could then be used to determine the time necessary for the desired degree of destruction of any given concentration of spores.

Spore counts obtained after boiling samples of the inoculated substrate for five minutes and spore counts obtained on samples drawn from the "tank" when the holding temperature was reached are recorded in Tables

1 to 5. In spite of the fact that uniform dilutions of a given spore suspension were used as inocula in the tests, there was appreciable variation in the "boiled five minutes" counts. The "temperature up" spore count was frequently greater than the "boiled five minutes" count, indicating either that the lethality during the time required to reach the holding temperature was less than the lethality of the five-minute heat treatment at 100°C.(212°F.) or that heat activation of the spores occurred during the coming-up time. The "temperature up" spore count was used as the original spore concentration in determining the percentage of survivors at the various time intervals in order to present data obtained during sustained heating at a uniform temperature.

TABLE 9

Destruction curve characteristics for Thermophilic Anaerobe No. 3814
Organism: T.A. No. 3814 (3-5-36) 1st centrifuge; 55°C.(131°F.) incubation.

Substrate	Quantitative in Norit-treated tryptone agar shake tubes		Direct incubation in food		Calculated F and z value (by method of least squares) for 99.99% destruction		Run No.
	F	z	F	z	F	z	
Phosphate (pH 7.0)	3.6	12.1	.....	.....	2.2	12.1	85-2, 86-3, 87-3, 88-3
Green beans	5.0	12.1	5.0	12.1	3.3	12.1	91, 92-1, 93-1, 94-1
Frozen corn	3.6	10.7	3.6	10.7	2.4	10.7	99 to 102
Frozen peas	4.8	11.1	5.2	11.1	3.0	11.1	95 to 98
Spinach	5.4	12.8	5.4	12.8	3.8	12.8	103 to 106

The survivor curves obtained with Flat Sour No. 1518 more closely approached a straight line passing through the origin than did the curves obtained with the other organisms, but even the flat sour survivor curves usually showed variations from this condition, the majority exhibiting a very slow initial destruction rate followed by a more rapid rate (Figure 1). This type of curve was obtained with neutral phosphate buffer solution as well as with food products which showed a progressive drop in pH during the heating period (Figure 2). It is believed that the initial slow rate of destruction is due to a preponderance of highly resistant spores in the flat sour spore crops or to heat activation overbalancing the destruction of the less heat resistant spores.

The majority of the survivor curves obtained with Putrefactive Anaerobe No. 3679, Thermophilic Anaerobe No. 3814, and *Clostridium botulinum* No. 62-A and No. 213-B exhibited a fairly rapid initial destruction rate followed by a slower rate (Figures 3 and 4). In our opinion this is due to the presence of a low percentage of highly resistant spores in these crops so that the spores of high resistance dominate the survivor curve only after an appreciable heating period. The marked increase in the slope of the survivor curves obtained with *Clostridium botulinum* No. 62-A after the destruction of 99.99% of the spores may be due to the presence of a

very low percentage of highly resistant spores (Figure 5). In some tests with Putrefactive Anaerobe No. 3679 and *Clostridium botulinum* No. 62-A, the survivor curve appeared sigmoidal, with a rapid initial destruction rate followed by a slower destruction rate and ending with a rapid rate (Figure 6). It is possible that the apparent rapid final destruction rate

TABLE 10

Destruction curve characteristics for Flat Sour Organism No. 1518
Organism: F.S. No. 1518 (3-8-35) 55°C.(131°F.) incubation.

Substrate	Quantitative in B.C.P. agar plates		Qualitative		Direct incubation in food		Calculated F and z value (by method of least squares) for 99.99% destruction		Run No.
	F	z	F	z	F	z	F	z	
Phosphate (pH 7.0)	25.3	16.5	.....	.....	.....	.....	16.4	16.5	107 to 110 107-1 to 110-1 109-2, 110-2, 110-3 Special high Special low
Asparagus	25	19.5	25	19.5	.....	.....	21	19.5	125 to 128
Green beans	25	17.8	.....	.....	.....	.....	19.8	17.8	113 to 115, 115-1, 116-1
Corn, frozen	36	21.4	.....	.....	36	21.4	29.6	21.4	153 to 156
Corn puree, frozen	36	17.8	.....	.....	36	17.8	28.7	17.8	169 to 172
Peas, fresh	26	16.9	26	16.9	.....	.....	20.2	16.9	117 to 120
Peas, frozen	44	19.8	.....	.....	44	19.8	30.9	19.8	117-1 to 120-1
Pea puree, frozen	36	19.9	.....	.....	21	19.9	25.4	19.9	165 to 168
Pumpkin puree	21	23.2	.....	.....	.....	.....	17.5	23.2	161 to 164
Shrimp	32	15.6	32	15.6	.....	.....	19.5	15.6	121 to 124
Spinach	30	20.8	30	20.8	.....	.....	24.7	20.8	141 to 144
Sweet potatoes (7.5% syrup)	28	20.5	28	20.5	.....	.....	22.1	20.5	133 to 136, 136-1
Sweet potatoes (30% syrup)	29	21.2	29	21.2	.....	.....	25.5	21.2	137 to 140

is the result of culturing limitations upon the recovery of the very few surviving spores.

Attempts to express the survivor curve as a single straight line resulted in wide variation of the best experimental D values for heat resistance tests which were identical save for the original concentration of spores (Figures 7 and 8). Unrecognized factors may be responsible for the deviation of the survivor curves from the linear relationship and the wide variation of D values derived from similar tests. Since the use of D values did not promise increased accuracy over the use of recovery endpoints in deter-

mining the slope *(z)* of the thermal death time curve, this method was not used in developing the data reported in Tables 1 to 11. The use of percentage destruction times appeared to be preferable to the use of endpoints since *z* variation resulting from erratic spore recoveries near the endpoint was minimized. The *z* values determined in this manner may be utilized to obtain the *F* value for any given spore concentration by appli-

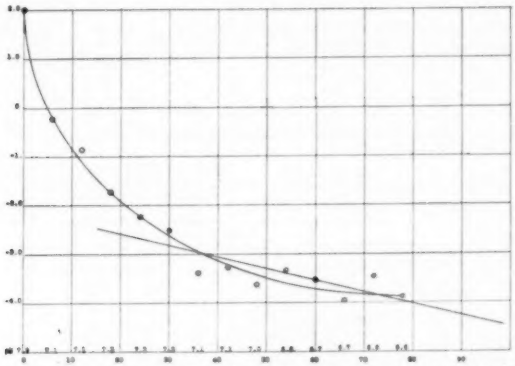

FIGURE 5. Survivor curve of *Cl. botulinum* 62-A in corn. Heated at 106°C.(222.8°F.). Run No. 74.

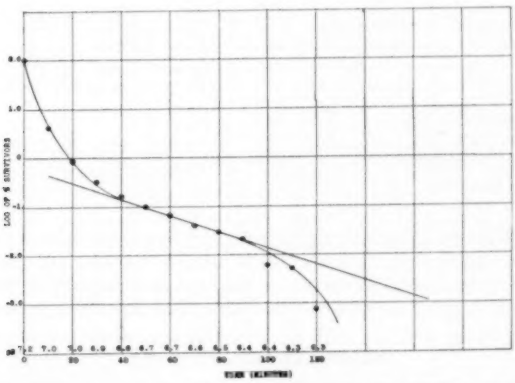

FIGURE 6. Survivor curve of Putrefactive Anaerobe No. 3679 in corn. Heated at 108°C.(226.4°F.). Run No. 158.

cation to spore recovery endpoints determined accurately at one or two temperatures.

The percentage destruction points for *z* value determination were selected so as to give due importance to the most heat-resistant spores, and the original spore concentrations were sufficiently high for possible

direct application of the endpoint data to the determination of processes. The calculated z values (Table 11) obtained in these studies could be applied satisfactorily to the quantitative spore recovery endpoints in most instances (Figures 9 and 10). The erratic nature of recoveries of *Clostridium botulinum* near the endpoint was such as to minimize the usefulness of either D values or percentage destruction points in determining more accurately the slope (z) of the thermal death time curve.

It is noted (Figure 9) that food-tube spoilage endpoints were appreciably lower than quantitative subculture endpoints for P. A. No. 3679 heated in green beans. Inspection of Tables 6 to 8 reveals that the inhibitive effect

TABLE 11

Effect of substrate on slopes of destruction curves

Product	Calculated z values for destruction of				
	99.99% P. A.	99.999% *Cl. botulinum* No. 62-A	99.999% *Cl. botulinum* No. 213-B	99.99% T. A.	99.99% F. S.
Phosphate	21.4 to 23.8	20.9	17.9	12.1	16.5
Asparagus	.....	.....	.....	.....	19.5
Beans, green	16.9	20.3	21.7	12.1	17.8
Beans, green, puree	26.6	.....	.....	.....	.....
Corn, yellow (frozen)	18.1[1]	17.7	.....	10.7	21.4
Corn, yellow, puree (frozen)	15.4	.....	.....	.....	17.8
Peas (fresh)	24.0	.....	.....	.....	16.9
Peas (frozen)	18.8	.....	.....	11.1	19.8
Pea puree (frozen)	21.2	.....	.....	.....	19.9
Pumpkin puree	.....	.....	.....	.....	23.2
Shrimp	21.4	.....	.....	.....	15.6
Spinach	18.3	19.4	.....	12.8	20.8
Spinach puree	22.9	.....	.....	.....	.....
Sweet potatoes (7.5% syrup)	18.7	.....	.....	.....	20.5
Sweet potatoes (30% syrup)	.....	.....	.....	.....	21.2
Sweet potatoes (50% syrup)	17.9	.....	.....	.....	.....

[1] Based on 99.999%.

of semi-acid products on putrefactive anaerobes is such as to make z values based on complete destruction of little use in determining the processes required for commercial sterility. In Table 9, it is noted that none of the products investigated showed an inhibitive effect upon the growth of T. A. No. 3814.

PRACTICAL IMPLICATIONS

Since the basic requirement for any canned food process is that it be sufficient to destroy the most heat-resistant organism which, by its survival, may result in a hazard to health, it is felt that minimum processes for low-acid canned products should be determined from F and z values required for the complete destruction of significant numbers of *botulinum* spores when heated in the product. The spore destruction rate studies on *botulinum* (Tables 2, 3, 7, and 8) indicate that the percentage destruction technique of determining the z value for *botulinum* does not show good

correlation with the z value determined by quantitative subculture, spore recovery endpoints.

Significant variation occurred in the z values obtained for different organisms when heated in the same product. This indicates that F and z values determined for a given spoilage organism in a particular product should be used in calculating the basic, minimum process for the prevention of spoilage by the given organism. When an organism which is not of health significance is concerned, product quality considerations may be

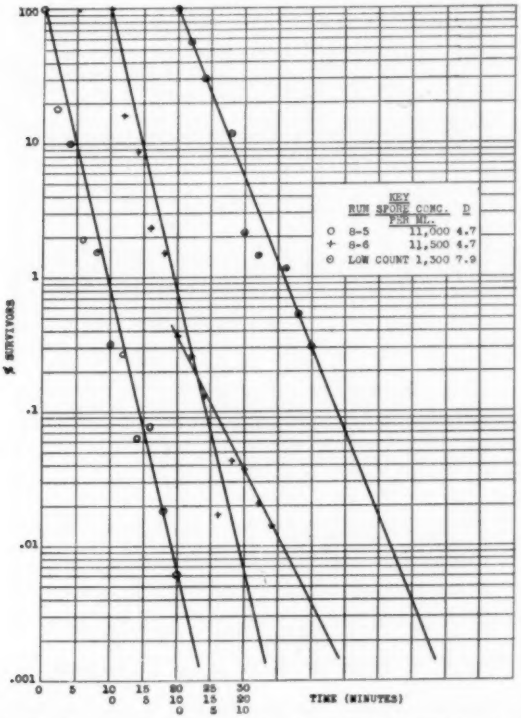

FIGURE 7. Survivor curves of Putrefactive Anaerobe No. 3679 in phosphate buffer at pH 7.0. Heated at 115°C.(239°F.).

such as to warrant the calculation of the process required for commercial sterility on the basis of F and z values obtained on direct incubation, that is, food-tube or T.D.T. can spoilage endpoints.

The most heat-resistant flat sour and thermophilic anaerobic types are generally obligate thermophiles, and commercial sterility may be obtained by proper cooling of the processed product and subsequent storage at temperatures below the thermophilic growth range. On the other hand,

Putrefactive Anaerobe No. 3679 is typical of the non-toxic, heat-resistant, mesophilic anaerobes which grow at normal storage temperatures, and commercial sterility with respect to non-toxic putrefactive spoilage, therefore, depends upon product inhibition. The F and z values reported for P.A. No. 3679 (Table 6) demonstrate that semi-acid products, such as green beans and sweet potatoes, exhibit an inhibitory effect upon the growth of the most heat-resistant surviving spores.

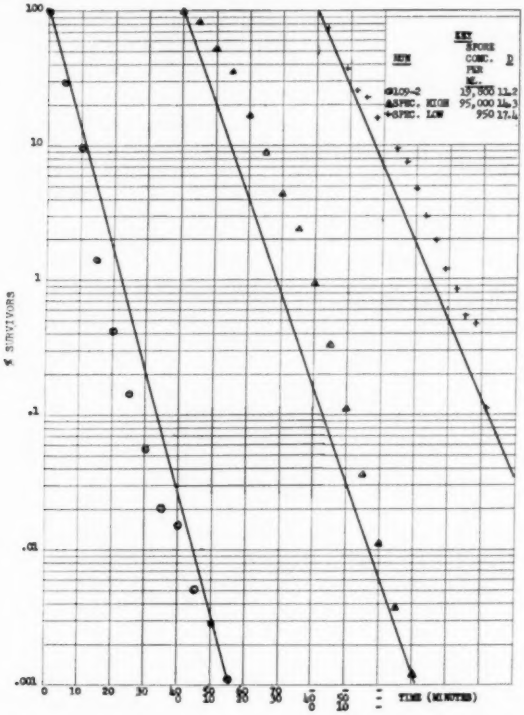

FIGURE 8. Survivor curves of Flat Sour No. 1518 in phosphate buffer of pH 7.0. Heated at 117°C.(242.6°F.).

The z values obtained for the destruction of P.A. No. 3679 in various products (Table 11) ranged from a low of 15.4 for corn puree to a high of 26.6 for green bean puree. A calculated process becomes longer as the z value is decreased, and the size of the process increment depends upon the total lethal requirement *(F)* and the way in which the product heats, that is, by convection or conduction. For example, a process for a No. 2 can of cream style corn (a conduction-heating product) to provide an F of 5.6 on the basis of z 18 is 96.1 minutes at 115.6°C.(240°F.) when the initial

product temperature is 60°C.(140°F.), while the process on the basis of z 15 is 101.7 minutes and on the basis of z 26 is only 85.1 minutes. The average z obtained on P.A. No. 3679 for the products investigated was 20.4, while only two z values (15.4 for corn puree and 16.9 for green beans) were more than 0.1°F. below 18. From this, it appears that the application of z 18 [which was originally established by Esty and Meyer *(6)* as the slope for spores of *Clostridium botulinum* when heated in neutral phosphate

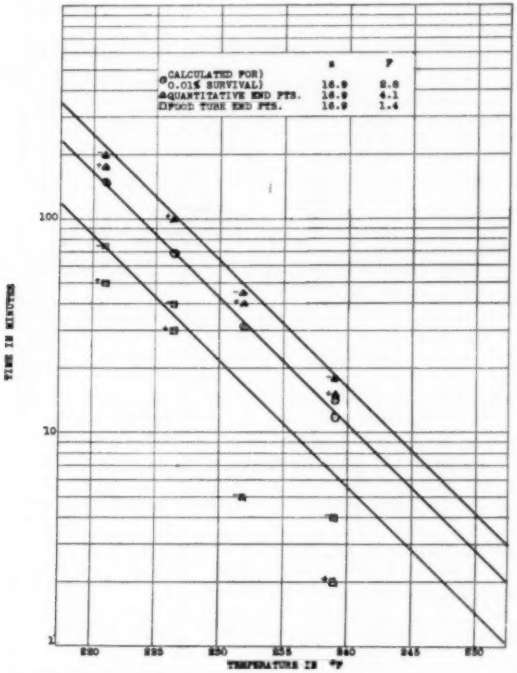

FIGURE 9. Thermal death time curves of Putrefactive Anaerobe No. 3679 in green beans. Runs Nos. 35, 36, 37-2, 38, 38-1.

solution] to the calculation of processes protective against non-toxic putrefactive spoilage has resulted in the proper process, or a process time slightly longer than necessary, for most products.

SUMMARY

Spore destruction rate tests were made on heat-resistant spores produced by organisms of significance to the canning industry. The spores were heated in the "tank" apparatus in substrates prepared from fresh or frozen foods, and the number of surviving spores was determined by culturing interval samples in favorable, quantitative media. Commercial

sterility endpoints were determined by culturing a portion of each interval sample in sterile tubes of the food under test.

All of the survivor curves obtained could not be represented as single straight lines passing through the origin, and the nature of the curves indicated that the spores in the suspensions under study were not of uniform resistance to heat. Times for a selected percentage destruction of spores were taken from the survivor curves and used to determine the slope of the thermal death time curve by the method of least squares. Thermal

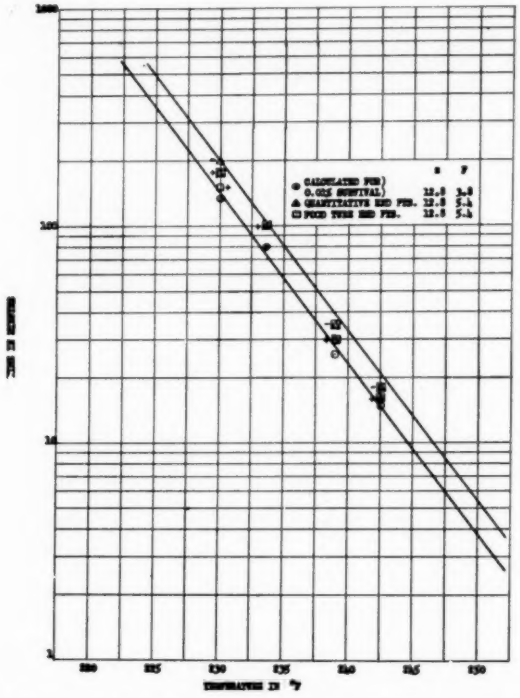

FIGURE 10. Thermal death time curves for Thermophilic Anaerobe No. 3814 in spinach. Runs Nos. 103-106.

death time slopes determined in this manner could be applied satisfactorily to the quantitative spore recovery endpoints in most instances, and slope variation resulting from erratic spore recoveries near the endpoint was minimized. The erratic nature of recoveries of *Clostridium botulinum* spores near the endpoint minimized the utility of either *D* values or percentage destruction points in determining more accurately the slope of the thermal death time curve for this organism.

Semi-acid products (green beans and sweet potatoes) inhibited to some extent the spore germination of putrefactive organisms, but showed no inhibitive effect on the germination of spores of a thermophilic anaerobe.

Thermal death time slope values determined for Putrefactive Anaerobe No. 3679 indicated that a z of 18°F. is a valid approximation for the calculation of processes protective against spoilage by this organism, while results on Thermophilic Anaerobe No. 3814 indicated that a z of 10°F. to 12°F. should be used to calculate processes protective against thermophilic anaerobic spoilage.

Acknowledgment

The authors gratefully acknowledge the helpful suggestions of J. R. Esty and C. T. Townsend, of this Association; F. C. Baselt and H. A. Benjamin, of the American Can Company Research Division; A. E. Stevenson, of the Continental Can Company Research Department; C. Olin Ball, of the Department of Food Technology, New Jersey Agricultural Experiment Station; O. B. Williams, of the University of Texas; and C. R. Stumbo, of the State College of Washington, in the preparation of this manuscript.

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APPENDIX

Pork broth medium. Trim the fat from fresh pork shoulder or loin. Grind the lean pork and mix with water (1 lb. of pork to 1 liter of water). Boil for 1 hour. Add NaOH so that pH of mixture will be 7.4. Remove the meat from the hot mixture by means of a press, such as a hand fruit press. Break up the cake of meat so it can be dried for later use. Cool the pork extract and place in a refrigerator so that the fat, when cold, can be easily removed by hand.

After the fat has been removed, make up the pork extract to volume. Add the following ingredients to 1,000 ml. of extract:

Difco peptone	5.0 g.
Difco tryptone	1.5 g.
Dextrose	1.0 g.
K_2HPO_4	1.25 g.

Dissolve and adjust to pH 7.4. The medium can be stored in liter flasks for immediate use or in No. 2 cans for later use. Autoclave 30 minutes at 15 p.s.i.g. pressure.

Pork agar medium. Add 1.5% agar to the pork broth medium.

Liver broth medium. Mix chopped beef liver with water in the proportion of 500 g. to 1,000 ml. Boil slowly for 1 hour, adjust to approximately pH 7.2, and boil for an additional 10 minutes. Pass the boiled material through a cheesecloth and make the broth up to 1,000 ml. To the broth add 10 g. of peptone and 1 g. of K_2HPO_4. In tubing, introduce one-half inch of the previously boiled ground beef liver into the tube and add the broth. The final pH after sterilization should be about 7.0.

Casein digest medium. Make up as much 1% Na_2CO_3 solution as you want medium. To this, add 10% casein, and heat in a water bath until the casein is well in suspension. Cool to about 40°C.(104°F.) and add 0.66 g. pancreatin per liter (trypsin is satisfactory). Distribute in flasks for digestion, using 15 ml. chloroform or toluene per liter for a preservative. Incubate overnight (approximately 18 hours) at 37°C.(98.6°F.). Boil off the preservative and acidify strongly with 5*N*. HCl to pH 5.6-5.2. Filter. Neutralize with 5*N*. NaOH. Add 0.5% Liebig's beef extract and 0.5% Na_2HPO_4. Make up to volume and check reaction to pH 7.2-7.6. Autoclave for 45 minutes at 15 p.s.i.g. pressure.

Norit-treated tryptone agar. Filter a 1% Difco tryptone 0.5% Norit broth by suction through filter paper. Add 0.125% K_2HPO_4 to the filtered broth and adjust to pH 7.0. Add 1.5% agar and tube in deep shake tubes. Autoclave for 30 minutes at 15 p.s.i.g. pressure.

EFFECTS OF FOUR COOKING PRESSURES ON LOCALLY GROWN BROCCOLI: COOKING TIME, PALATABILITY, ASCORBIC ACID, THIAMINE, AND RIBOFLAVIN

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(Manuscript received March 13, 1951)

The purpose of this study was to determine the effects of increased temperature, with increased pressure, on total cooking time, palatability, ascorbic acid, thiamine, and riboflavin retention in locally-grown broccoli. It is known that in pure solutions ascorbic acid, thiamine, and riboflavin are affected in the presence of heat. The cooking methods used besides boiling at atmospheric pressure were 5, 10, and 15 p.s.i.g.

Brinkman, Halliday, Hinman, and Hamner *(2)* reported that fresh broccoli was definitely inferior in palatability when cooked in the pressure saucepan.

The ascorbic acid content of broccoli has been reported in the literature to range from 68 mg. per 100 g. by McHenry and Graham *(11)* to 128 ± 6 by VanDuyne, Bruchart, Chase, and Simpson *(16)* and 128 ± 7 by Collings *(5)*. The range of retention of ascorbic acid during cooking of broccoli has been reported from 38% by Burrell and Elbright *(3)* to 69 ± 3 to $77 \pm 6\%$ by Collings *(5)*.

The thiamine content of broccoli has been reported in the literature from as low as 0.08 mg. per 100 g. by Morgan, MacKinney, and Gailleau *(12)* to 0.202 by Banton *(1)*. Retentions in drained cooked broccoli ranged from 87% to complete retention as reported by Collings *(5)* depending upon the proportion of water used at 0, 5, or 15 lb. pressure. Oser, Melnick, and Oser *(15)* reported 95% retention in broccoli cooked by boiling.

The range of riboflavin content of broccoli has been reported from 0.176 mg. per 100 g. by Hodson *(9)* to 0.320 by Oser, et al. *(15)*. Reported retentions in drained cooked broccoli range from 83% by Oser, et al. *(15)*, and from 83% to complete retention by Collings *(5)* depending upon the method of cooking. Collings also reported some increases of riboflavin during cooking.

EXPERIMENTAL PROCEDURES

The broccoli was of Calabrese variety and was grown at Geneva, New York. One-half ton of 4-8-12 fertilizer was used per acre. The broccoli was dusted three times during the season with a rotonal duster. It was delivered to the laboratory the afternoon it was cut, and was placed in the hydrator of a refrigerator. It was cooked the following morning.

Preliminary preparation. The broccoli was trimmed and washed in running cold water. It was drained in a shallow pan lined with cheese cloth. The stalks were cut into four-inch lengths, and the larger stalks were split lengthwise.

Cooking. For cooking at each of the four pressures, a four-quart alumnium pressure saucepan having a diameter of eight inches and a depth of five inches was used. In the studies employing atmospheric pressure the vent in the cover was left open. In order to determine when the air was exhausted from the pan, and also to check the

pressure with the internal temperature of the pan, a thermometer was placed in a rubber stopper which was inserted in the cover of the pressure saucepan after the automatic safety disc was removed. The heat was controlled by the use of a manometer *(8)*.

In each study, 300-g. lots of broccoli, enough for four servings, were used. The broccoli was dropped into a weighed amount of boiling, unsalted tap water. The amount of water used was such that the amount left at the end of cooking was approximately the same regardless of the cooking method used. Previous work in this laboratory has shown that the loss of water-soluble vitamins to solution is chiefly dependent upon the proportion of water (up to the point of immersion) to vegetable left after cooking. Two hundred g. of water were used in the tests at atmospheric pressure and 145 g., in the tests employing the other three pressures. The amount of water used with atmospheric pressure was more than that usually recommended because the cover used allowed steam to escape constantly during cooking.

The gas burner was turned down a predetermined amount as soon as the desired pressure was reached. In each study, the broccoli was cooked until it was "just done." This time had been determined in preliminary studies (Table 1). At the end of cook-

TABLE 1

The time required to cook broccoli at four different pressures

Cooking pressure, lb. (gauge)	Cooking times required			
	To reach 100°C.[a]	From 100°C. to pressure	At pressure[b]	Total[c]
	min.	*min.*	*min.*	*min.*
(Atmospheric)	3¼	....	10	13¼
5	3	1	3	7
10	3	1¾	1¼	6
15	3	2	½	5½

[a] Time for the water to return to the boil after the broccoli was added.
[b] At the end of the times given, cold water from the tap was allowed to run over the cover. As soon as the pressure was brought to zero the cover was removed and the broccoli sampled.
[c] Time from placing the broccoli into the boiling water until removing it from the heat.

ing, the temperature was reduced immediately to approximately 100°C. by holding the pan under cold running tap water. The total weight was determined and the broccoli drained 45 seconds. The drained broccoli was weighed, and the weight of the cooking water was determined by difference.

In the four cooking methods, the weight of the drained cooked broccoli ranged from 311±2.0 to 319±4.4 g. and of the cooking water from 64±4.6 to 83±5.5 grams. Each cooking study was repeated five times.

Sampling. A series of cookings, including one at each of the four pressures, was made on the same day. Raw samples were taken for vitamin analysis just prior to the first cooking and after two of the four cookings were completed. Broccoli in 200-g. lots was quickly chopped on a wooden board with a knife having a chromium blade. After the chopped broccoli was mixed, duplicate samples were weighed for analysis. Ten g. of raw and cooked vegetable and 2 ml. of cooking water were analyzed for ascorbic acid; 25 g. of raw and cooked vegetable and 10 g. of cooking water were analyzed for thiamine and riboflavin. Sampling was done in a room having controlled lighting.

Palatability. Broccoli cooked by each method was judged for appearance, texture, and flavor by six competent judges. Maximum score for each of the three quality factors was five. A summation score of 15 was equivalent to excellent; 12, good; 9, fair; 6, poor; and 3, very poor.

Vitamin analysis. Reduced ascorbic acid was determined by the indophenol-xylene extraction method of Nelson and Somers *(14)*. Thiamine was determined by the modified thiochrome method of Conner and Straub *(6)* as adapted for vegetables by Moyer and Tressler *(13)*. Riboflavin was determined by the method of Conner and Straub *(7)* as modified by Mackinney and Sugihara *(10)*.

Statistical treatment. The percentage retention of ascorbic acid, thiamine, and riboflavin in the broccoli as served was calculated on the total weights of the raw and

of the cooked broccoli in each cooking. A statistical analysis was made to determine whether the observed differences were significant or merely attributable to experimental variation.

RESULTS AND DISCUSSION

Cooking times. Little difference in total cooking time was required at the three pressures, from 5½ to 7 minutes (Table 1). From the management standpoint the difference in time was insignificant. Zero lb. pressure required approximately twice as long, 13¼ minutes, as did cooking under pressure.

Palatability. Broccoli cooked by all four methods was considered acceptable by the judges. The average summation scores for the broccoli cooked by 0, 10, and 15 p.s.i.g. were the same, 12 (Table 2). The average summation score for the broccoli cooked at 5 p.s.i.g. was 13. Neither the above differences nor the differences in scores for color, texture, and flavor of the broccoli cooked by the four methods were significant. As great a difference occurred between the scores of the several judges as between the scores for the broccoli cooked by the different methods.

TABLE 2

The palatability scores for broccoli cooked at four pressures[a, b]

Cooking pressure lb. (gauge)	Color score		Texture score		Flavor score		Total score	
	mean	*range*	*mean*	*range*	*mean*	*range*	*mean*	*range*
(Atmospheric)	4	3-5	4	4-5	4	2-5	12	10-14
5	4	4-5	4	2-4	5	4-5	13	11-14
10	4	4-5	4	2-5	4	3-4	12	10-14
15	5	4-5	3	2-4	4	3-5	12	10-14

[a] Scores of six judges.
[b] The maximum score for each of the three quality factors was five.

The finding is not in agreement with that of Brinkman et al. *(2)* who reported that broccoli cooked in a pressure saucepan at 15 p.s.i.g. was definitely inferior to that cooked in an open kettle or in a waterless cooker. Their broccoli was purchased on the retail market and required a longer cooking than did the fresh broccoli used in this study. At the elevated temperatures of pressure saucepan cooking any over-cooking is quite disastrous to the palatability of a vegetable, particularly a brassicaceous one.

Vitamins. The average vitamin content per 100 g. of raw broccoli was 131 mg. (116 to 160) of ascorbic acid, 0.14 mg. (0.08 to 0.20) of thiamine, and 0.22 mg. (0.17 to 0.26) of riboflavin. No significant differences in the percentage retentions of ascorbic acid, thiamine, or riboflavin occurred in the broccoli.

The retentions of ascorbic acid in the broccoli cooked by the four methods ranged from 74 ± 13.8 to $83 \pm 5.5\%$, and in the cooked vegetable plus cooking water from 86 ± 13.5 to $94 \pm 7.6\%$. Retentions of thiamine in the drained cooked broccoli ranged from 80 ± 13.7 to $95 \pm 14.4\%$, and in the cooked vegetable plus cooking water from 98 ± 9.8 to $112 \pm 13.1\%$. Retentions of riboflavin in the drained cooked broccoli ranged from 84 ± 9.4 to $102 \pm 14.2\%$, and in the vegetable plus cooking water from 95 ± 9.7 to $113 \pm 15.3\%$ (Table 3). These findings are similar to those of Causey and

TABLE 3

The effect of four cooking pressures on the retention of ascorbic acid, thiamine, and riboflavin in broccoli[f]

Cooking pressure, lb. (gauge)	Ascorbic acid			Thiamine			Riboflavin		
	Drained broccoli	Water	Total	Drained broccoli	Water	Total	Drained broccoli	Water	Total
	%	%	%	%	%	%	%	%	%
(Atmospheric)	74±13.8	12±1.6	86±13.5	80±13.7	22±4.1	102±12.4	95±26.7	14±4.6	109±30.4
5	81± 8.3	10±1.1	91± 9.0	82±13.9	17±3.0	99±12.9	101±12.4	11±2.0	112±14.3
10	83± 5.5	11±2.4	94± 7.6	76± 7.8	22±5.4	98± 9.8	84± 9.4	11±2.0	95± 9.7
15	77±10.6	10±2.5	87±11.4	95±14.4	17±3.6	112±13.1	102±14.2	11±1.4	113±15.3

[f] No significant differences were noted. Percentage retentions were calculated on the total weight of the broccoli and of the cooking water at the beginning and end of cooking; each figure is the mean value of five replications.

Fenton *(4)* on ascorbic acid and folic acid in frozen broccoli cooked at 0, 5, 10, and 15 p.s.i.g., and of Collings *(5)* on thiamine and riboflavin in broccoli, cooked at 0, 5, and 15 p.s.i.g. Evidently, pressure alone was not a determining factor in retentions in any of the above studies.

Ascorbic acid, thiamine, and riboflavin apparently were little if any affected by any of the cooking pressures used in this study. What loss occurred was to the cooking water. This tends to confirm previous findings that the amount of cooking water is probably the most important factor in the retention of the water-soluble vitamins.

A 100-g. serving of broccoli, cooked by any one of the methods used, provided all of the 75 mg. of ascorbic acid, three-fourths of the 1.5 mg. of thiamine, and practically all of the 1.8 mg. of riboflavin recommended as the daily allowance by the National Research Council. The content of a 100-g. serving of the broccoli ranged from 97 to 109 mg. of ascorbic acid, from 0.10 to 0.14 mg. of thiamine, and from 0.17 to 0.22 mg. of riboflavin.

SUMMARY

Broccoli was cooked in home amounts at 0 lb. pressure (boiling) and at 5, 10, and 15 lb. pressure. The amount of water used was such that approximately the same amount was left at the end of each cooking, regardless of pressure. Total cooking times, palatability, and ascorbic acid, thiamine, and riboflavin retentions were determined.

Differences in total cooking times at 5, 10, and 15 p.s.i.g. were not sufficient to recommend one cooking time over another. Zero lb. (atmospheric) pressure required about twice as long as did cooking under the other pressures.

Little difference in palatability scores resulted. All of the broccoli was considered acceptable.

No significant differences occurred in the percent retentions of ascorbic acid, thiamine, or riboflavin in the broccoli cooked at the four pressures. The vitamins were either in the vegetable or in the cooking water.

It would seem that a choice of one cooking procedure over any other used in this study for broccoli would be a matter of personal preference.

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HEAT INACTIVATION OF THE BASIC AMINO ACIDS AND TRYPTOPHAN[a]

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Revived interest in the amino acid content of foods and feedstuffs and in the role of amino acids in nutrition has come since microbiological and chromatographic technics have been developed for amino acid determination. Many values for the amino acid content of human foods and animal feedstuffs have been reported recently, but little is still known about the availability of these amino acids or factors affecting their availability.

Protein-bound lysine has been known for several years to be inactivated by heat so that it is not nutritionally available *(11, 21)*. Recent evidence *(1, 2, 9, 14)* has indicated that other amino acids might also be inactivated by heat treatment of proteins. Soy bean meal that was autoclaved for 4 hours at 15 p.s.i.g. pressure or for 1 hour at 130°C. did not support normal growth of chicks fed diets supplemented with the known vitamins *(2, 9)*. However, supplementation of the diets with lysine and methionine restored growth to normal *(2, 14)*. Some destruction of protein-bound lysine and cystine in the heat-treated soy bean meals was observed *(10, 18)*.

The present investigation was the outgrowth of an attempt by the authors to determine the cause of lysine, methionine, and cystine inactivation when soy bean meal was autoclaved. Using isolated soy bean protein and sucrose (because there is no glucose in soy bean meal) studies were made of the inactivation of lysine *(4)*; methionine *(5)*; and cystine *(8)*. It was believed desirable to repeat the studies with lysine extending them to include arginine, histidine, and tryptophan, and to also determine if soy bean oil or any of the carbohydrates found in soy bean meal besides sucrose are involved in the heat inactivation of these amino acids.

EXPERIMENTAL

The procedures used and definition of heat destruction and heat inactivation are similar to those used in the methionine study *(5)*. Soy bean meal, soy bean protein, and mixtures of 8 g. of soy bean protein and 2 g. of sucrose, glucose, dextrin, agar-agar, gum arabic, or soy bean oil were autoclaved for 4 hours at 15 p.s.i.g. pressure. Unautoclaved samples of each were used as controls except in the case of lysine where the unautoclaved protein served as control for all.

Acid hydrolysates were prepared by autoclaving 1.0 g. of the material with 20 ml. of 20% hydrochloric acid for 6 hours at 15 p.s.i.g. pressure. Enzymatic digests were prepared by hydrolyzing with the enzymes trypsin and erepsin in vitro *(3)*. Alkaline digests for tryptophan determinations were prepared by autoclaving 1.0 g. of the material with 0.2 g. of cysteine hydrochloride and 20 ml. of 4 *N*. sodium hydroxide for 16 hours at 15 p.s.i.g. pressure *(13)*. All amino acids were determined by microbiological

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assay using the media described by Sauberlich and Baumann *(19)*. *Lactobacillus casei* was the organism used for arginine, *Leuconostoc mesenteroides* for lysine, *Streptococcus faecalis* for histidine, and *Lactobacillus arabinosus* for tryptophan.

RESULTS

The data obtained for lysine are presented in Table 1, those for arginine in Table 2, those for histidine in Table 3, and those for tryptophan in Table 4. Tryptophan liberated from soy bean meal, soy bean protein, the protein plus glucose, and the protein plus sucrose by alkaline digestion is compared in Table 5 with tryptophan liberated by digestion with trypsin and erepsin in vitro, and by pepsin, trypsin, and erepsin digestion in vitro *(3)*. Alkaline digestion usually liberated the most tryptophan, although pepsin, trypsin, and erepsin digestion in vitro liberated almost as much from the unautoclaved samples.

DISCUSSION

Sucrose, stachyose, arabans, galactans, and dextrin have been reported as the principal carbohydrates of soy bean meal *(17, 20)*. Unfortunately, stachyose was not available for use in this experiment. Gum arabic was used as a representative araban and agar-agar as a galactan. Previous studies, by Evans and Butts *(4)*, of lysine inactivation were made with sucrose as the only supplement added to the soy bean protein. The data presented in Table 1 confirm the earlier conclusions that two types of

TABLE 1

Effect of autoclaving soy bean oil meal or soy bean protein on the inactivation of lysine in the protein[b]

Expt. No.	Soy bean product	Non-protein material added	Treatment	Acid hydrolysis		Enzyme hydrolysis	
				Lysine content	Lysine destroyed	Lysine content	Lysine inactivated
				%	% of total[c]	%	% of total[d]
1	Oil meal	.....	.....	3.14	0	1.27	0
	Oil meal	.....	Autoclaved	1.85	41	0.52	59
	Protein	.....	.....	5.00	0	3.17	0
	Protein	.....	Autoclaved	4.86	3	2.34	26
	Protein	Sucrose[e]	Autoclaved	2.50[f]	50	0.66[f]	79
	Protein	Dextrin[e]	Autoclaved	4.35[f]	13	2.10[f]	34
	Protein	Agar-agar[e]	Autoclaved	4.48[f]	10	2.22[f]	30
	Protein	Gum arabic[e]	Autoclaved	4.80[f]	4	2.54[f]	20
2	Protein	.....	.....	4.63	0	2.89	0
	Protein	.....	Autoclaved	4.38	5	1.97	32
	Protein	Glucose[e]	Autoclaved	2.27[f]	51	0.86[f]	70
	Protein	Sucrose[e]	Autoclaved	2.50[f]	46	0.90[f]	69
	Protein	Soy bean oil[e]	Autoclaved	3.89[f]	16	1.55[f]	46
	Protein	Mixture[g]	Autoclaved	2.00[f]	57	0.78[f]	73

[b] 10-g. portions were autoclaved for 4 hours at 15 p.s.i.g. pressure. Decreases in the lysine content of the soy bean materials as determined on the acid hydrolysate after autoclaving are considered to be due to destruction of lysine, those determined on the enzymatic hydrolysate as due to "inactivation," including destruction.

[c] Total lysine is considered to be the lysine content of the unautoclaved material determined after acid hydrolysis.

[d] Total lysine is considered to be the lysine content of the unautoclaved material determined after enzymatic hydrolysis.

[e] Eight g. of soy bean protein and 2.0 g. of non-protein material.

[f] Lysine content in the soy bean protein.

[g] Five g. of soy bean protein, 1.0 g. of sucrose, 1.0 g. of dextrin, 1.0 g. of agar-agar, 1.0 g. of gum arabic, and 1.0 g. of soy bean oil.

inactivation of lysine occur when soy bean meal is drastically autoclaved. One is actual destruction caused by a reaction of protein-bound lysine (probably the free epsilon amino group) with sucrose or the reducing sugar glucose formed from sucrose during autoclaving. The other is inactivation caused by a reaction of protein-bound lysine with other constituents of the soy bean protein to form a linkage from which lysine is liberated in a form available to *Leuconostoc mesenteroides* by acid hydrolysis but not by in vitro digestion with trypsin and erepsin. Destruction of protein-bound lysine was as great when soy bean protein was autoclaved with glucose as when it was autoclaved with sucrose. There was some destruction of lysine when the protein was autoclaved with dextrin, agar-agar, or soy bean oil, but not nearly as much as when the protein was autoclaved with sucrose or glucose. No more of the lysine was inactivated (not including that destroyed) in soy bean protein autoclaved with one of the carbohydrates than in the protein autoclaved by itself.

In preliminary work, the authors *(6)* indicated that when soy bean protein was autoclaved with sucrose, protein-bound arginine was destroyed in a manner similar to lysine destruction but that there was no inactivation when the protein was autoclaved alone. The data presented in Table 2

TABLE 2

Effect of autoclaving soy bean oil meal or soy bean protein on the inactivation of arginine in the protein[b]

Soy bean product	Non-protein material added	Acid hydrolysis		Enzyme hydrolysis	
		Arginine content	Arginine destroyed by autoclaving	Arginine content	Arginine inactivated by autoclaving
		%	% *of total*	%	% *of total*
Oil meal	.....	5.02	35	4.71	36
Protein	.....	8.47	0	6.61	9
Protein	Sucrose	8.89	42	6.77	62
Protein	Glucose	8.89	42	6.46	60
Protein	Dextrin	8.86	9	6.60	14
Protein	Agar-agar	8.65	4	6.66	16
Protein	Gum arabic	8.79	3	6.69	11
Protein	Soy bean oil	8.89	2	8.72	11
Protein	Mixture	9.82	47	10.06	63

[b] For details of the experiments see footnotes [b] to [e], Table 1. Substitute "arginine" for "lysine."

confirm the preliminary results with soy bean protein and sucrose and extend them to soy bean meal and to a mixture of soy bean protein and glucose. Addition of dextrin, agar-agar, gum arabic, or soy bean oil to the protein before autoclaving had no appreciable effect on arginine destruction or "inactivation." The data indicate that the addition of either sucrose or glucose to soy bean protein before it is autoclaved may increase the amount of arginine inactivated to a greater extent than the amount destroyed. Arginine and lysine behaved similarly in that over 40% of each was destroyed when a mixture of soy bean protein and sucrose was autoclaved. They differed in that 30% of the lysine and only 9% of the arginine were inactivated when soy bean protein was autoclaved. Although

both lysine and arginine have free-NH_2 groups to react with glucose, the group of arginine is part of a guanidino radicle which does not appear to react with other constituents of the soy bean protein in a manner similar to the epsilon amino group of lysine.

Histidine also contains extra nitrogen and is basic in reaction. Its extra nitrogen is combined in the imidazol group. Evidently the imidazol group is not as reactive with sucrose as are the amino and guanidino groups, because only 20% of the histidine was destroyed when soy bean protein was autoclaved with sucrose or glucose compared to the destruction of over 40% of the lysine and arginine (Table 3). Twenty percent of the histidine

TABLE 3

Effect of autoclaving soy bean oil meal or soy bean protein on the inactivation of histidine in the protein[1]

Soy bean product	Non-protein material added	Acid hydrolysis		Enzyme hydrolysis	
		Histidine content	Histidine destroyed	Histidine content	Histidine inactivated
		%	% of total	%	% of total
Oil meal	.....	1.46	17	1.06	33
Protein	.....	2.45	6	2.12	20
Protein	Sucrose	2.31	20	1.96	68
Protein	Glucose	2.34	20	1.74	65
Protein	Dextrin	2.35	9	1.96	35
Protein	Agar-agar	2.34	10	2.08	27
Protein	Gum arabic	2.36	7	2.02	26
Protein	Soy bean oil	2.27	8	1.80	23
Protein	Mixture	2.33	24	1.96	60

[1] For details of the experiments see footnotes [b] to [g], Table 1. Substitute "histidine" for "lysine."

was inactivated when soy bean protein was autoclaved. The amount of histidine inactivated was greater when the protein was mixed with sucrose or glucose before it was autoclaved. Dextrin caused some increase in amount inactivated when mixed with the soy bean protein before autoclaving, but agar-agar, gum arabic, and soy bean oil were without effect. Evidently the imidazol group of histidine reacts with some constituent of soy bean protein and with sucrose or glucose to form compounds resistant to in vitro trypsin and erepsin digestion but the compounds formed are hydrolyzed by acid digestion to give microbiologically active histidine.

When soy bean meal or soy bean protein plus sucrose or glucose were autoclaved tryptophan was destroyed to about the same extent as histidine but there was no inactivation of tryptophan when the protein was autoclaved alone (Table 4). Tryptophan behaved similarly to histidine in that it was inactivated when the protein was autoclaved with sucrose or glucose but not with any of the other carbohydrates. A slight inactivation came from the oil. When soy bean protein was autoclaved, the indole group of tryptophan appeared to react similarly to the imidazol group of histidine in some respects but to differ in others. It would appear that the –NH– portion of the imidazol and indole groups might have been responsible for the slight destruction and greater inactivation of tryptophan and histidine that occurred when soy bean protein was autoclaved with sucrose

TABLE 4

Effect of autoclaving soy bean oil meal or soy bean protein on the inactivation of tryptophan in the protein[j]

Soy bean product	Non-protein material added	Alkaline hydrolysis		Enzyme hydrolysis	
		Tryptophan content	Tryptophan destroyed	Tryptophan content	Tryptophan inactivated
		%	% of total[k]	%	% of total
Oil meal	.....	0.57	16	0.46	20
Protein	.....	1.03	7	0.80	0
Protein	Sucrose	1.01	17	0.77	49
Protein	Glucose	1.05	27	0.63	48
Protein	Dextrin	1.06	11	0.71	0
Protein	Agar-agar	1.04	9	0.81	12
Protein	Gum arabic	1.06	7	0.79	1
Protein	Soy bean oil	0.91	0	0.80	16
Protein	Mixture	0.98	4	0.86	46

[j] For details of experiments see footnotes [b] and [d] to [g], Table 1. Substitute "tryptophan" for "lysine."

[k] Total tryptophan is considered to be the tryptophan content of the unautoclaved material determined after alkaline hydrolysis.

or glucose. The inactivation of histidine occurring when the protein was autoclaved by itself appears to have been caused by some other group or combination of groups in the imidazol radicle.

Table 5 presents a comparison of the tryptophan content of some of the soy bean samples as determined after alkaline digestion, digestion with pepsin, trypsin, and erepsin in vitro, and after trypsin and erepsin digestion in vitro. The tryptophan contents of the pepsin, trypsin, and erepsin digest of unautoclaved soy bean meal or protein were similar to or higher than that of the alkaline digest. Addition of glucose or sucrose to soy bean protein before in vitro enzymatic digestion decreased the amount of tryptophan liberated. Prior autoclaving decreased the amount of tryptophan liberated by alkaline digestion but decreased to a greater extent that liberated by pepsin, trypsin, and erepsin digestion in vitro. Less tryptophan was liberated by trypsin and erepsin digestion in vitro than by pepsin, trypsin, and erepsin digestion in vitro of unautoclaved materials. Either

TABLE 5

Liberation of tryptophan from soy bean meal or protein by different methods of hydrolysis

Soy bean product	Non-protein material added	Treatment	Tryptophan content after hydrolysis with		
			Alkali	Pepsin-trypsin-erepsin	Trypsin-erepsin
			%	%	%
Oil meal	.....	.....	0.57	0.66	0.46
Oil meal	.....	Autoclaved	0.48	0.35	0.37
Protein	.....	.....	1.03	1.00	0.80
Protein	.....	Autoclaved	0.96	0.80	0.81
Protein	Sucrose	.....	1.01	0.84	0.77
Protein	Sucrose	Autoclaved	0.84	0.45	0.39
Protein	Glucose	.....	1.05	0.91	0.63
Protein	Glucose	Autoclaved	0.77	0.39	0.33

method liberated a similar amount from the autoclaved materials. Enzymic digestion in vitro with pepsin, trypsin, and erepsin has been used by Evans, Davidson, Bandemer, and Butts *(7)* to digest egg samples for tryptophan determinations, because higher values for tryptophan were obtained than had been reported by workers using alkaline hydrolysis.

As shown by the decreased tryptophan content of the alkaline hydrolysates in Table 5, some tryptophan was destroyed when soy bean meal or the protein were autoclaved. Some tryptophan was tied-up by autoclaving so that it was not released by pepsin, trypsin, and erepsin digestion in vitro. In the latter case the tryptophan was apparently bound in the unautoclaved protein by a linkage requiring pepsin to open it. The tryptophan contents of the trypsin and erepsin digests of the autoclaved and unautoclaved protein and the pepsin, trypsin, and erepsin digest of the autoclaved protein were similar but were lower than the tryptophan content of the pepsin, trypsin, and erepsin digest of the unautoclaved protein. Apparently autoclaving of the protein changed the linkage in some manner so that pepsin would no longer break it.

We worked only with soy bean meal and soy bean protein as sources of protein, but reports of other workers indicate that similar data might have been obtained using other proteins or concentrates. Riesen, Clandinin, Elvehjem, and Cravens *(18)* observed that when soy bean meal was overheated, a destruction of lysine, arginine, and tryptophan, but not of hystidine occurred. There was no destruction of lysine, arginine, histidine, or tryptophan when casein was autoclaved for 20 hours *(12)*, but when casein was refluxed in a glucose solution, losses of all took place *(15)*. Similar losses occurred when soy globulin was refluxed with glucose *(16)*.

SUMMARY

In these experiments only one method of heating, namely, autoclaving for 4 hours at 15 p.s.i.g. pressure was used. Three types of inactivation of the basic amino acids and tryptophan occurred when a complex protein concentrate, such as soy bean meal, was autoclaved. These were 1, a reaction of the protein-bound amino acid with either glucose or sucrose to destroy the biological activity of the amino acid; 2, a reaction of the protein-bound amino acid with some other constituent of the protein to form a linkage resistant to in vitro digestion with trypsin and erepsin but not to acid hydrolysis; and 3, a reaction of the protein-bound amino acid with sucrose or glucose to form a linkage with properties similar to type 2. Dextrin, agar-agar, gum arabic, or soy bean oil played little or no part in the inactivation.

Lysine inactivation was of types 1 and 2, arginine was of types 1 and 3, histidine was of all three types, and tryptophan was 1 and 3. Lysine appeared to suffer the greatest destruction and histidine and tryptophan the least.

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SPOILAGE BACTERIA IN TOMATO PRODUCTS[a,b]

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The prevention of spoilage is a most important consideration in the processing of tomato products. Outbreaks of spoilage in tomato paste, pulp, and juice occur almost every year and occasionally the losses sustained reach serious proportions. However, many packers regard some of these losses as almost inevitable and take no concerted action to correct faulty conditions.

It has long been recognized that species of *Lactobacilli* and yeasts are frequently responsible for swelled cans of tomato pulp and related products and the classic studies reported by Pederson *(7, 9)* several years ago contain comprehensive reviews of the literature dealing with these organisms. More recently, Jones *(6)* also ascribed species of *Lactobacilli* as the cause of spoiled tomato products of high viscosity.

The marketing of tomato juice characterized by a peculiar off-taste and odor known as "flat-sour," is detrimental to the brand name of the processor and to the industry because a natural liking for tomato juice may be lost. The organism causing this type of spoilage was first isolated and described by Berry *(2)* in 1931 and named *B. thermoacidurans*. Later, Stern *(10)* showed the fastidious growth requirements of this organism in the laboratory, and Cameron and Esty *(5)* reported a wide variation in the relative heat resistance of various strains and the number of spores required to initiate spoilage. Becker and Pederson *(1)* found physiological characteristics common to several strains and Pederson and Becker *(8)* demonstrated experimentally in the laboratory that the development of flat-sour juice was influenced by the pH level of the product.

The work reported in this paper was undertaken during 1949 and 1950 to investigate spoilage potentialities, and the outbreaks of spoilage in four plants processing tomato juice and in two plants packing tomato paste and pulp. Two of the four juice processors employed flash pasteurization procedures while the other two firms in the study used conventional low temperature processing methods. Both firms packing tomato paste and pulp filled at 88°C.(190°F.) followed by air cooling or retorting and water cooling.

The work of the first season entailed a detailed bacteriological examination of fully-processed production samples which were taken two or three times a day from the six plants. The cans were shipped promptly to a storage warehouse where they were kept at 0°C.(32°F.) until examined. The following season, direct investigations of the processing lines were conducted as the bacteriological findings of the previous year warranted.

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[a] Presented before the Eleventh Annual Meeting of the IFT, New York, N. Y., June 19, 1951.

[b] A report of a study sponsored jointly by the Ontario Research Council and a group of Ontario canners.

BACTERIOLOGICAL PROCEDURES

The cultural methods employed during the investigations were basically those recommended by Cameron and Bohrer *(4)* and included bacterial plate counts on dextrose tryptone agar (Difco) with incubation at 37°C.(98.6°F.) and 55°C.(131°F.). Plates of proteose peptone acid agar (supplied as Thermoacidurans agar Difco) were also incubated at 55°C.(131°F.). All readings were made after 48 hours of incubation. The presence of gas-forming anaerobes in the products was determined by examining daily for a four-day period tubes of inoculated tryptone broth which had been stratified with agar seals. At the commencement of the studies, tubes containing tomato paste, pulp, and juice were incubated at 37°C.(98.6°F.) and 55°C.(131°F.) for 7 days but this period was later increased to 15 days. Samples of high viscosity were diluted with physiological saline to permit even distribution of colonies and ease of handling. All counts were calculated from 1-ml. samples. Microscopic and organoleptic examinations of all products were made before and after incubation and a Beckman glass electrode meter was employed in the determination of pH. Many cultures obtained from a total of 605 samples of juice, paste, and pulp were examined and set aside for subsequent identification studies.

During the second season, samples were collected in the plants by the bacteriological staff and the specimens were analyzed within a few hours. The procedures generally followed were those referred to above with the supplemental use of tomato juice agar (Difco) for the examination of paste and pulp line samples.

Colonies picked from plates of proteose peptone agar were tested for the characteristics given by Becker and Pederson *(1)* for *B. thermoacidurans* while other organisms were checked for characteristics listed by Breed, Murray, and Hitchens *(3)*.

Sterile 0.1 *N*. citric acid was used for adjusting the tomato juice to various pH levels required in the study. Spore suspensions of the organisms were prepared by heating the washed growths from slants of acid-agar for a period of seven minutes in flowing steam.

During the heat studies, an Aminco oil bath, equipped with an electric agitator and stirrer was employed. Tubes which had been tested were cooled rapidly by immersion in ice water.

RESULTS AND DISCUSSION OF PASTE AND PULP EXAMINATIONS

The results of analyses conducted on tomato paste and pulp samples packed by the two processors on the same days in 1949 are shown in Table 1. The findings obtained from these plants followed the same pattern throughout the season and will not be given here. Contrary to what might have been expected, substantial spoilage was experienced by Plant A, although an examination of the products packed by this firm showed relatively few organisms. Some of the samples spoiled on incubation in the laboratory and many swelled cans were reported by the firm. An examination of the spoiled samples showed that in all instances *Lactobacilli* species were responsible, and that the predominating organism in the spoiled samples was a strain of *Lactobacillus fermentii* Beijerinck. All organisms isolated were readily destroyed when inoculated into tomato juice of pH level 4.2 and held at a temperature of 70°C.(158°F.) for one minute. However, in tomato paste some organisms caused sporadic spoilage until heated for two minutes at this temperature. This occurrence was undoubtedly due to the poor thermal conductivity of the product.

Line surveys in Plant A showed that *Lactobacilli* species were not present in the products at the filler bowl but appeared in empty cans passed through the closing machine. This fact was noted at the beginning of the second season's pack and the subsequent inauguration of a stricter control program in the plant eliminated losses.

Significant spoilage did not develop in the products from Plant B despite the incidence of high plate counts at the time of packing. Line

TABLE 1

Comparison of bacteriological findings on tomato paste and pulp packed by two processors on the same days during 1949

Plant	Day of test	Average plate counts, bacteria per ml.		Gas-forming anaerobes per 3 ml. tryptone broth, 37°C.(98.6°F.)
		Dextrose tryptone, 37°C.(98.6°F.)	Proteose peptone, 55°C.(131°F.)	
A	1	1	0	Absent
	2	3	0	Absent
	3	1	0	Absent
	4	0	0	Absent
	5	2	0	Absent
	6	0	0	Absent
	7	1	0	Absent
	8	0	0	Absent
	9	0	0	Absent
	10	2	0	Absent
	11	6	0	Absent
	12	0	1	Absent
	13	0	0	Absent
	14	0	0	Absent
	15	0	0	Absent
B	1	315	45	Present
	2	10	85	Present
	3	6	20	Present
	4	3	1	Absent
	5	4	25	Absent
	6	2	75	Absent
	7	30	15	Absent
	8	6	6	Absent
	9	30	2,800	Present
	10	85	35	Present
	11	170	180	Absent
	12	65	5	Absent
	13	3	0	Absent
	14	30	40	Absent
	15	60	25	Present

examinations showed that both yeasts and *Lactobacilli* species were present on the underside of closing equipment immediately after a typical clean-up and empty cans passed through the closing machine during another run also showed high counts. As this firm retorted its products after closing, the *Lactobacilli* of low heat resistance and yeasts were almost invariably destroyed and the remaining mixed flora of other bacteria, although high in numbers, did not affect the keeping quality of the products. Despite the high counts also obtained from this plant by plating the paste and pulp on proteose peptone acid agar, flat-sour spoilage did not occur. Gas-forming organisms which grew in the tubes of tryptone broth produced butyric or cheesy odors but the growth of these bacteria was evidently inhibited in undiluted tomato paste.

The plates of dextrose tryptone agar which were incubated at 55°C. (131°F.) and tubes of the products which were incubated at 55°C.(131°F.) did not add any additional data which could be linked to the occurrence of spoilage.

Sometimes it was very difficult to detect occasional colonies of *Lactobacilli* species which grew on the dextrose tryptone agar plates. Tomato juice agar (Difco) used during the second year for the examination of paste and pulp samples showed the development of healthier cells and larger colonies.

RESULTS AND DISCUSSION OF TOMATO JUICE EXAMINATIONS

Only a few of the 253 samples of tomato juice examined showed counts of over five organisms per ml. when plated on the neutral dextrose tryptone agar. Thermophylic and mesophylic anaerobes were found in only seven of the cans examined and the presence of these organisms did not affect the keeping qualities of the juice. It will be noted in Table 2 that

TABLE 2

"Flat-sour" contamination in tomato juice packed during 1949

Plant	No. samples examined	Samples showing plate counts on proteose peptone		
		%	Average plate counts, bacteria per ml.	Average pH of juice
C	33	27.0	1.4	4.28
D	69	2.9	1.0	4.26
E	96	21.9	1.8	4.31
F	55	25.1	1.7	4.26

there was a pronounced variation in the incidence of acid-tolerant organisms found in the different packs. However, only Plant E reported any cans showing flat-sour spoilage and these were few in numbers. In fact, only one can was available for studies and although bacteria were present in microscopical preparations, no organisms were recovered on culturing the juice. It will be seen in Table 2 that the average pH value of the juice packed by this firm was the highest of all juices studied but that the average spore load of aciduric organisms was not appreciably greater than that found in the juices packed by the other canners.

The following season, when line examinations were conducted in Plants C and E, bacterial counts on dextrose tryptone agar indicated good sanitation in both instances (Table 3). Acid-tolerant organisms were destroyed

TABLE 3

Results of examinations in 1950 showing incidence of bacteria in tomato juice processing lines

Sampling location	Average plate counts, bacteria per ml.[c]			
	Plant C		Plant E	
	Dextrose tryptone	Proteose peptone	Dextrose tryptone	Proteose peptone
Pulp after heating	0	1	2	1
Juice after heating	0	0	....[d]	1
Juice at filler bowl	0	0	1	0
Processed juice	0	0	3	1

[c] The culturing temperatures were as given in Table 1.
[d] Number undertermined.

completely during processing in Plant C but in Plant E these organisms passed without any obvious "build-up," through from the tomato pulp into the processed juice. This firm was cautioned concerning the danger of flat-sour spoilage. As the season was well advanced, however, no steps could be taken to alleviate the danger at the time. It was also questionable whether flat-sour spoilage would occur as the spore load of aciduric organisms in the processed tomato juice was lower than that usually considered safe based on the information and published data which was currently available *(4)*.

Flat-sour spoilage of the tomato juice processed by this plant later proved to be substantial and did not show up until the pack had started to move from the warehouse. The examination of cans picked at random from codes which showed a high percentage of flat sours indicated that only a low spore load of aciduric bacteria was present (Table 4) but that

TABLE 4

Results of the random sampling of cans from Plant E which showed "flat-sour" spoilage

pH of juice	Average plate counts, bacteria per ml. on proteose peptone agar at 55°C.(131°F.)	Taste and odor
4.42	4	Normal
4.40	1	Normal
4.42	5	Normal
4.39	3	Normal
3.89	3	Strong flat-sour
4.41[e]	1	Normal
4.41	3	Normal
4.42	0	Normal
3.88	1	Strong flat-sour
4.39	4	Normal
4.39	0	Normal
4.40	4	Normal
4.42	2	Normal
4.01	1	Slight flat-sour
4.43	5	Normal
4.45	6	Normal
4.18	....[e]	Slight flat-sour
4.08	2	Slight flat-sour
4.43	4	Very slight flat-sour

[e] Too numerous to count without dilution.

the average pH level of the pack was considerably higher than had been noted previously (Table 2). Subsequent pH determinations on many cans of tomato juice packed in 1950 by the other three processors concerned, showed that the pH level of the juice from Plant E was outstandingly high (Table 5).

Two strains of an aciduric organism were recovered from the spoiled samples. Based on several cultures examined the physiological characteristics of the isolates agreed with those noted by Becker and Pederson *(1)* for *B. thermoacidurans*.

Heavy inoculations of spore cells made into tomato juice having a pH value of 4.12 failed to reproduce flat-sour spoilage but when inoculations were made into juice of pH 4.32, spoilage readily occurred. Vegetative cells caused spoilage in both sets of adjusted tomato juice but in the juice of the lower pH level, a full week of incubation was necessary before any evidence of flat-sour spoilage was detectable. In the more alkaline tomato juice, typical flat-sour characteristics were noted within a few days. It should be noted that when vegetative and spore cells of the organism were inoculated into tomato juice having pH values of 4.40 and 4.50 and heated for six minutes at 99°C.(210°F.), flat-sour spoilage occurred in all tubes following incubation. This treatment was more severe than the processing given the tomato juice of Plant E.

TABLE 5

pH values of tomato juice packed by four companies during 1950

Plant	Maximum pH	Minimum pH	Average pH
C	4.25	4.12	4.17
D	4.35	4.10	4.25
E	4.45	4.39	4.40
F	4.27	4.10	4.20

SUMMARY

1. A bacteriological survey of the total packs of six plants processing tomato products was made during 1949. Based on the findings obtained, a study of spoilage factors was carried out in the plants and laboratory during 1950.

2. High bacterial counts and the presence of butyric anaerobes in tomato paste and pulp were not indicative of the keeping qualities of these products.

3. Spoilage losses in tomato paste and pulp were caused by species of *Lactobacilli* and yeasts. Contamination by these organisms occurred after the filling operation. The organisms were relatively non-heat resistant and were destroyed by strict plant procedures.

4. A low spore load of *B. thermoacidurans* (Berry) in tomato juice caused serious commercial losses of flat-sour spoilage.

5. In practice and experimentally, the pH level of tomato juice influenced the development of flat-sour spoilage.

6. No evidence of a "build-up" of aciduric organisms was found in the lines of a company which experienced flat-sour spoilage.

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THE MICROBIOLOGY OF DEHYDRATED VEGETABLES[a]

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The present period of world unrest has stimulated general interest in the dehydration industry. Part of this renewed attention has been focused on the microbiological aspects of vegetable dehydration, a subject which received the first impetus as a result of World War I and was spurred to further advances during the period of World War II. It is the purpose of this paper to discuss some of the factors which determine the microbial populations of dehydrated vegetables; the relation of these factors to the types of microorganisms which may be used as indices of quality and sanitation; and to stress additional problems which need attention. The author's own experimental work will be integrated with information already published.

HISTORICAL CONSIDERATIONS

With one known exception, the investigations concerning the microbiology of dehydrated vegetables were stimulated by wartime production of these commodities. The investigators were primarily interested in the types of microorganisms which survived the dehydration process and in the various factors which influenced their survival. The most important investigations which have come to the attention of the author include those of Prescott *(14, 15)*; Prescott, Nichols, and Powers *(16)*; Clague *(3)*; Clague and Fuller *(4)*; Kintner and De Lay *(11)*; Jones *(9)*; Haines and Elliot *(8)*; and Jones and Pierce *(10)*.

The microbial populations of dehydrated vegetables. Independent studies on a large number of samples of various dehydrated vegetables have shown very few, if any, of them to be sterile. Furthermore, as is to be expected, because of the comparatively low acidity and sugar content as contrasted with fruits, the predominating populations of the dehydrated vegetables have been found to be bacteria. Prescott *(14)*, who was among the first to identify the types of microorganisms isolated from dehydrated vegetables, found the majority of the species to be common soil and waterborne bacteria of the genera *Bacillus* and *Pseudomonas*. Prescott also recovered molds rather frequently but found no yeasts. He identified the molds as species of *Aspergillus, Mucor, Penicillium, Spicaria, Sporotrichum, Trichoderma,* and *Herpocladiella*. This list of genera was enlarged in a subsequent publication *(16)* to include *Alternaria, Fusarium, Monilia,* and *Thamnidium*. Other investigators either did not encounter molds with the same frequency or did not attach the same significance to their presence, as did Prescott and his associates. However, they, like Prescott, always found a preponderance of bacteria and no yeasts.

[a] Presented at the Eleventh Annual Meeting of the IFT, New York, N. Y., June 19, 1951.

Other investigators extended the list of genera of bacteria first reported by Prescott. Coliform bacteria (genera *Escherichia* and *Aerobacter*) were isolated and identified by Clague *(3)*, Kintner and De Lay *(11)*, Jones *(9)*, Haines and Elliot *(8)*, and Jones and Pierce *(10)*. Haines and Elliot also identified additional types of bacteria isolated from a variety of dehydrated vegetables. These included the genera *Achromobacter, Bacillus, Clostridium, Micrococcus, Pseudomonas,* and *Streptococcus* (viridans and enterococcus groups). These latter investigators also found unidentified thermophiles occasionally and recovered unidentified molds and actinomyces from dried potatoes.

Bacteria capable of causing human infections or food poisoning (species of *Salmonella, Clostridium botulinum,* and *Micrococcus pyogenes* var. *aureus*) were not recovered from dehydrated vegetables, although serious attempts were made to isolate them. It appears, on the basis of these investigations, that bacteria originally derived from the soil or water apparently predominate the microbial populations of dehydrated vegetables. It is obvious, however, that other important genera of bacteria might be expected to predominate under certain conditions. In fact, although their presence was not reported earlier, the author has found species of *Lactobacillus* and *Leuconostoc* to dominate the populations of microorganisms in many samples of dehydrated vegetables.

Production factors which influence the microbial populations. Several factors in production have been reported to influence markedly the microbial populations of dehydrated vegetables. These factors include:

1. The microbial quality of the fresh produce.
2. The method of pre-treatment of the vegetables (peeling, blanching, etc.).
3. The time elapsed between preparation of the vegetables and start of the dehydration process.
4. The time involved in dehydration of the vegetables.
5. The temperature of dehydration.
6. The moisture content of the finished product.
7. The general level of sanitation in the dehydration plant.

The microbial populations of dehydrated vegetables are also influenced by such factors as the chemical characteristics of the vegetables; the presence or absence of antibiotic compounds in the vegetables; the effective use (correct loading) of the dehydrator; and the average "finishing" temperature of the vegetables at the end of the dehydration process. Although the effects of most of these factors have been recognized in a general way, many have received little or no consideration with respect to their effect on bacteria which may survive on dehydrated vegetables.

EXPERIMENTAL

Interest in the microbiology of dehydrated vegetables was stimulated by the occasional souring and fermentation of potatoes and onions which occurred during the process of dehydration. In the study of these spoilage problems methods for the examination of product samples and equipment surfaces were developed. These methods subsequently were used for general studies on the microbiology of vegetables.

Methods

It was desirable to use a method for the enumeration of microorganisms which was simple, required a minimum of equipment, saved a maximum of time, and still could be used in the laboratory or in the field with comparable accuracy. After experimentation with various procedures *(3, 9, 11, 14)*, the following method was adopted for the examination of all fresh, partially dried, or dehydrated vegetable samples:

1. A representative sample was collected under aspectic conditions.
2. A representative portion of this sample was weighed under aseptic conditions.
3. The weighed portion (10 g. if dried, 50 g. if fresh sample) was transferred to sterile water contained in wide-mouthed ground glass-stoppered pyrex bottles. In most instances the initial dilution was 1:10 but with powdered onions or "fines" of other vegetables the initial dilution was always 1:20.
4. The diluted sample was shaken steadily and vigorously for 10 minutes, preferably by a shaking machine, and then plated out in appropriate dilutions.

Four media were used for estimation of the various types of microorganisms. Levine's eosine methylene blue agar (Difco) was used for estimation of coliform bacteria. This medium is especially valuable for the rapid detection of *Escherichia coli* and related bacteria. Yeast water-glucose agar, prepared from fresh compressed yeast *(19)*, adjusted to pH 5.0-5.5 was used for estimation of lactic acid bacteria, yeasts and molds. Difco's dextrose tryptone agar (National Canners Association formula) fortified with 0.5% yeast extract (Difco) was selected for estimation of "total" numbers of organisms because it always gave higher counts than nutrient agar and other media frequently used for that purpose. This medium also permits ready estimation of acid-forming organisms. Nutrient agar (standard methods formula of the American Public Health Association) was also included because of its general use in survey work.

Equipment surfaces which contributed to contamination of vegetable particles were examined by the method already described by Vaughn and Stadtman *(21)*. Other conventional methods used will be cited below.

RESULTS

The souring of vegetables during dehydration caused considerable concern early in the period of conversion for increasing production of these products for the armed forces. The souring obviously was very undesirable; it not only increased the labor and time involved in quality inspection but, in cases of incipient souring, it also reduced the quality of the affected dehydrated vegetables to such an extent that they did not meet required standards of acceptability when rehydrated.

Souring During Dehydration

It was speculated that a combination of factors contributed to souring. It had been observed that spoilage most frequently occurred when the vegetables had been loaded onto trays in uneven clumps or piles or when the trays had been uniformly overloaded. Under such conditions, as will be shown, the vegetables did not dry at a normal rate and, because the evaporative cooling effect was pronounced for a longer period, the vegetables remained at a favorable incubation temperature for rapid development of spoilage organisms. Onions and potatoes were frequently soured under the conditions that existed during the early phases of conversion to wartime production, consequently they received the most attention.

Souring of onions. Soured onions have a characteristic sour odor and taste commonly associated with vegetable tissues which undergo lactic acid fermentaion. Their moisure content is excessive when they are removed from the tunnels. On further drying the characteristic sour odor fre-

quently disappears and discoloration (pinking) and taste have to be used as criteria of spoilage.

The bacteria which predominated the microbial populations of soured onions were found to be representative of the genera *Lactobacillus* and *Aerobacter*. The species included 20 cultures of *L. plantarum*, 5 cultures of *L. brevis*, 30 cultures of *A. aerogenes*, and 22 cultures of *A. cloacae*, all of which had been isolated, studied, and identified according to methods accepted in Bergey's Manual of Determinative Bacteriology *(1)*. There is some evidence to explain the failure to find significant numbers of other microorganisms in soured onions. It is known through the work of Sarti *(17)*, Brown *(2)*, Walker *(24)*, Walker, Lindegren, and Bachman *(25)*, Lovell *(12)*, Fuller and Higgins *(7)*, and Pederson and Fisher *(13)*, that the vapor and juice of fresh onions have a germicidal effect on a variety of bacteria. In our own experiments it was found that fresh onion juice, sterilized by filtration, killed cultures of *Bacillus subtilis*, *Escherichia coli*, *Candida krusei*, and *Saccharomyces cerevisiae* but did not prevent the growth of *L. plantarum*, *L. brevis*, or *A. aerogenes*. Thus, the failure to find many aerobic bacilli and other soil or water-borne bacteria or yeasts in soured or good quality dehydrated onions is, in part at least, due to the toxicity of certain constituents of the fresh onion.

Sliced fresh onions prepared for dehydration seldom carry less than 10,000 viable microorganisms per gram, and usually the count ranges between 50,000 and 100,000 viable organisms per gram of fresh sample. Even under the most optimum conditions of handling and dehydration the reduction in numbers seldom exceeds 50% in the finished product. Rapid dehydration of freshly sliced onions is desirable and, as indicated by the data shown in Table 1, the maximum holding time should not exceed 2

TABLE 1

Bacterial count of onions sliced and left standing before dehydration[b]

Standing time, hours	Bacteria per gram, on fresh weight basis	
	E.M.B. agar	Yeast glucose agar
0	5,925	28,500
2	22,000	35,250
4	39,000	106,000
8.5	310,000	675,000

[b] Net weight of onions on car, 580 lbs.; equal to tray load of 1.24 lbs. per sq. ft. Air temperature at start of experiment: 40°C.(104°F.)

hours before the onions are started through the dehydration process. Tray loading also is an important factor in preventing undesirable increases in numbers of viable microorganisms, as shown by the data collected in Table 2. The importance of optimum tray loading is emphasized by the thermocouple measurements shown in Figure 1, in which the effect of loading on product temperature is clearly illustrated.

Proper plant sanitation practices also are required to assist in the prevention of excessive contamination of onions. Equipment surfaces must be kept clean and free from excessive microbial populations because onions

are not blanched and are not finished at temperatures high enough to effect marked reductions in the microorganisms which may be present at the start of the process.

Spoilage of potatoes. Noticeably spoiled dehydrated potatoes were always discolored and partially disintegrated. The heavily infected pieces (slices, dices, or strips) took on a chalky appearance and some had "eyes" indicative of gas formation as shown in Figure 2. On rehydration, the individual pieces crumbled badly, acquired an unnatural grayish color and emanated a spoiled odor. Furthermore, the taste was somewhat putrid, rather than sour as was so characteristic of soured onions.

TABLE 2

The effect of car loading on survival of microorganisms during dehydration of onion slices

Car number[g]	Car load	Tray load	Bacterial counts (dry weight basis)[c]			
			Before drying	After primary tunnel	After secondary tunnel[d]	After bin[d]
	lbs.	*lbs./sq. ft.*				
1	435	0.93	365,000	312,000	70,200	108,000[e]
2	630	1.35	180,000	248,000	59,700,000	13,020,000 (composite)[f]
					62,000,000	24,700,000 (wet)
3	650	1.39	299,000	245,000	3,980,000	10,000,000 (composite)[f]
					14,800,000	12,000,000 (wet)

[c] Counts made on yeast infusion, glucose agar.
[d] The apparent increase in numbers is partly the result of sampling error, which for obvious reasons, is quite high with onion slices, but also the result of multiplication of the bacteria.
[e] This is a composite sample of a uniformly dry car.
[f] The composite samples include dry and wet slices which had soured.
[g] Total drying time of 24¾ hours. Finishing air temperature of primary parallel airflow tunnel 130°F.(54.4°C.); finishing air temperature of secondary counter-current airflow tunnel 143°F. (61.7°C.); finishing air temperature of bin 115°F.(46.1°C.).

The bacteria which predominated the microbial populations of spoiled potatoes were found to be starch-fermenting species of the genera *Aerobacter* and *Bacillus*. The species identified by methods already cited included 24 cultures of *Aerobacter aerogenes*, 10 cultures of *Bacillus subtilis*, 5 cultures of *B. megatherium*, and 11 cultures of *B. cereus*, of which 8 cultures were probably *B. cereus* var. *mycoides* because of their distinctive rhizoid growth on nutrient agar.

Blanching, to destroy enzyme activity, and the higher finishing temperature of 148-155°F.(64.5-68.5°C.) used for potatoes made it possible to produce dry potatoes with very low total counts as compared with onions. Actual microbial spoilage of potatoes during dehydration was eliminated when proper attention was given to optimum loading of trays and cars so as not to exceed the optimum drying capacity of the tunnels. With the introduction of inplant, break-point chlorination to control microbial contamination of equipment surfaces, recontamination of the blanched potatoes was greatly reduced.[h] Introduction of sulfiting to improve quality of the dehydrated potatoes also inhibited rapid multiplication of microorganisms during dehydration. The use of bin drying to insure uniform moisture

[h] Data which emphasized the need for recontamination control was published by Vaughn, Stadtman, and Kueneman *(23)*.

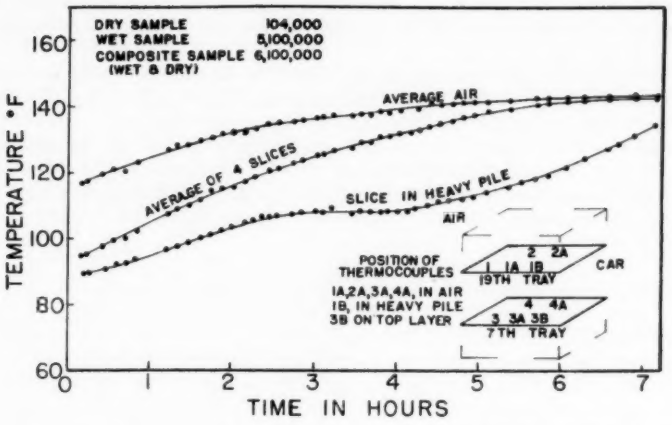

FIGURE 1. Thermocouple measurements to show effect of car loading on finishing temperature of dehydrated onions.

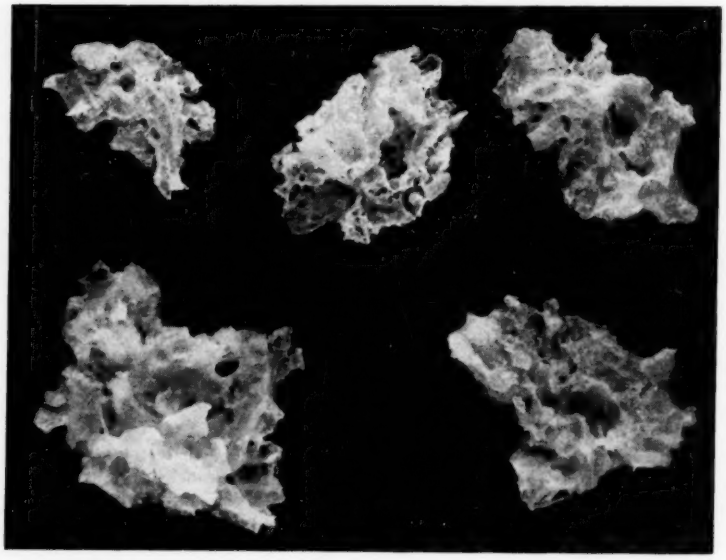

FIGURE 2. Clumps of soured, partially decomposed, dehydrated potatoes. Note evidence of extensive gas formation.

content also was effective in further reduction of microorganisms. Similar observations were made when dehydration of cabbage and carrots was investigated.

Microorganisms as Indices of Quality of Various Dehydrated Vegetables

One of the problems concerned with the study of the microbiology of vegetables was the possible use of microorganisms as indices of quality. To avoid arbitrary and unattainable indices, a thorough knowledge of the microbial populations of dehydrated vegetables was required before any suggestions could be made concerning tolerances which might be useful under accepted conditions of production. As already shown in connection with the study of onions and potatoes, the nature of the product, its preparation, and temperature of its dehydration markedly influence the final microbial population of the finished product.

The variations in "total" counts of viable microorganisms of various dehydrated vegetables certified as acceptable for use by the armed forces are shown in Table 3. The extremely high maximum counts obtained for

TABLE 3

Variations in "total" counts of commercial samples of dehydrated vegetables certified as acceptable, 1944-1945

Product	No. of samples	Total count		Maximum finishing temp., °F.
		Minimum	Maximum	
Onions	53	12,600	7,500,000	140
Potatoes	31	<100	22,800,000[1]	155
Sweet potatoes	18	< 10	47,000	165
Cabbage	20	<100	60,000	145
Rutabagas	2	40,000	92,000	150
Carrots	11	<100	40,000	160
Beets	4	60,000	520,000[1]	155

[1] These samples were taken from 5 gallon lots produced in adapted "fruit" tunnels.

onions and potatoes indicate the need for careful study of the relation of the product and its processing to its microbial population. These high maximum counts represented only a single sample of each product.

A study of the predominant populations of these two samples was quite interesting. Lactic acid bacteria *(Lactobacillus)* comprised at least 90% of the "total" population of the onions; the majority of the remainder was coliform bacteria *(Aerobacter)*. The population of the potato sample was almost completely predominated by the single species *Aerobacter aerogenes*, as only about 5% of the "total" count consisted of other microorganisms. These two samples had undergone marked microbial decomposition or had been heavily contaminated during processing. In either case, much lower maximum "total" were attainable, especially in the case of the blanched potatoes. The potatoes had been dehydrated in adapted fruit tunnels and, although the plant was very clean and sanitary, the counts were always high because of the slow, inefficient dehydration. Under more favorable conditions in other plants, the maximum "total" counts always were much lower and never exceeded 100,000 viable organisms per gram, even when the sanitary conditions of the plants were not satisfactory.

The more efficient dehydration practices permitted the finishing temperature of the potatoes to approach that of the dehydrator air and, as a result, a lethal temperature was operative for a longer period of time. Therefore, it is proper to question the value of "total" counts of blanched dehydrated vegetables as indicators of sanitation and quality.

The author is of the opinion that no "total" microbial tolerances for blanched, dehydrated vegetables should be established without thorough evaluation of the conditions under which the products were produced and the effect of the finishing temperature on the survival of the microorganisms which might have caused incipient spoilage during the earlier stages of dehydration. Blanching, if sufficient to inactivate enzymes, will reduce the contamination of the fresh produce to an insignificant figure (*3, 11, 9,* and Table 4). Therefore, the obligation should be to maintain the "total" counts at a uniformly low level during subsequent steps of production, and not to depend on the finishing temperature to destroy the microorganims which might otherwise be used as indices.

TABLE 4

Reduction in numbers of microorganisms affected by commercial steam blanching of vegetables to be dehydrated

Product	Total count per gram (wet weight)[j]		Percent reduction in total count
	Before blanching	After blanching	
Beets	3,150,000	<100	>99.9
Potatoes	36,000	< 10	>99.9
Potatoes	7,050,000	170	>99.9
Potatoes	2,110,000	10	>99.9
Potatoes[k]	235,000	30,500	87.0
Potatoes	28,000,000	25	>99.9
Potatoes[l]	3,600,000	2,496,000	33.5
Potatoes[m]	15,000	67,000	Increase
Potatoes	480,000	100	>99.9
Potatoes	435,000	<100	>99.9
Potatoes	137,000	<100	>99.9
Potatoes	75,000	< 10	>99.9
Carrots	435,000	60	>99.9
Cabbage	42,500	25	>99.9

[j] Tryptone glucose agar with brom cresol purple.
[k] Sample taken from trays as it was impossible to obtain sample at the end of the blancher belt.
[l] Sample taken from first potatoes through blancher after a shutdown period.
[m] The increase is the result of mechanical inefficiency of the blancher. Increases in counts have been observed a number of times with other blanchers of known inefficiency.

It is very important that arbitrary tolerances be avoided for unblanched, dehydrated vegetables such as onions or garlic. These products have been produced primarily for use as ingredients in other food products. Consequently, the use may, in greater or lesser measure, dictate the desired tolerance. However, until these products have been properly evaluated under commercial conditions of production, tolerances must be made with reservations. For example, at one time a commercial tolerance for dehydrated onions required that these products contain no *Escherichia coli*. As already stated, unknown constituents of the onion are very toxic

to *E. coli*. In proof that this tolerance was of no value, to date the author and his associates have not recovered *E. coli* from samples of dehydrated onions, although over 300 samples have been investigated over a period of seven years.

DISCUSSION

Several problems related to the microbiology of dehydrated vegetables need further attention. As already stressed, there is a need for more information on the thermal changes which occur in the various products during dehydration. Unless more information on product finishing temperatures is accumulated and correlated with surviving microbial populations it would appear unwise to attempt to establish "total" count tolerances or to use various groups of bacteria as possible indices of quality or sanitation for finished dehydrated vegetables. There also is a serious lack of information concerning the thermal resistance of these bacteria at temperatures used for dehydration.

Sulfite solutions used commercially to retard discoloration of cabbage and potatoes were found to reduce the microbial populations found on these dehydrated products. Preliminary experiments with the epoxides showed that ethylene oxide could not be used to significantly reduce the microbial contamination on completely dehydrated vegetables. However, if the vegetables were treated before dehydration or when partially dehydrated (25 to 30% moisture), marked reductions in numbers of microorganisms could be obtained with this fumigant at atmospheric temperature and pressure. The desirability of extending these observations on the use of sulfites and the epoxides as well as other compounds is obvious.

Finally, it is believed that a better understanding of the microorganisms which may survive on dehydrated vegetables is needed. The survey work to date has given little attention to the types of yeasts which may be found on dehydrated vegetables. New selective media which might be useful for isolation of specific bacteria or groups of bacteria which are found on dehydrated vegetables have not been tested. Buffered boric acid lactose broth *(20b)* might be useful for detection and presumptive identification of *Escherichia coli*. Sorbic acid, liver infusion agar, or other sorbic acid media should be useful for detection and enumeration of the lactic acid bacteria *(20a)*.

SUMMARY

The microbiology of dehydrated vegetables was discussed in relation to the factors which influence the microbial populations of the finished products.

The souring of onions and potatoes during dehydration was described in detail to show the necessity for optimum tray loading and its relation to the finishing temperature of the dehydrated products.

The possible use of microorganisms as indices for quality of dehydrated vegetables was discussed in relation to the need for attainable tolerances. The necessity for obtaining additional information was stressed.

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SOME OBSERVATIONS ON THE BROWNING REACTION[a,b,c]

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The interaction of carbohydrates with amines and amino acids, usually referred to as the Maillard *(8)* or browning reaction, long important because of its role in the naturally occurring phenomenon of non-enzymic browning, has recently assumed new interest as a result of increased production of dehydrated and other new types of processed foods. Although the reaction in certain instances is beneficial or even essential, in many natural products it leads to the development of undesirable color, flavor, and insolubility, making their long storage impossible.

Since the early work of Maillard *(8)* little has been learned of the chemical nature of the changes which take place. Study of model systems of simple sugars and amine, imine, or amido type compounds has appeared most likely to afford insight into the initial phases of the reaction since condensation products structurally similar to carbohydrate oximes and hydrazones may be postulated as intermediates in the development of the complex darkly colored reaction products. Mitts and Hixon *(9)* and Kuhn and Birkofer *(4)* have described the preparation of a number of such N-glycosides or glycosylamines. More recently Hurd and Kelso *(3)*, by using desoxyaldopentoses, have demonstrated the importance of the α-hydroxyaldehyde function in the browning reaction. In the present investigation, undertaken simultaneously, a similar conclusion was reached through a study of the reactions of certain amines with 2,3,4,6-tetramethyl-D-glucose, glyceric aldehyde, glycoaldehyde, and a number of glycolaldehyde ethers. The effect of the basicity of various amines was also observed in the present work.

EXPERIMENTAL

The glucose used in the following studies was anhydrous D-glucose supplied by the Corn Products Company. Anhydrous Dextrose of the Eastman Kodak Company was found to be equally satisfactory. With the exception of p-nitroaniline and the amino acids, the amines were carefully distilled through a short Vigreaux column. The amino acids (Eastman Kodak Company white label grade) were used as obtained. The p-nitroaniline (Eastman white label) were recrystallized from aqueous alcohol.

Browning of aqueous solutions of D-glucose and amines. To 20-ml. portions of 0.01 *M.* solutions of D-glucose were added 20-ml. portions of 0.1 *M.* solutions of the

[a]The work herein reported was carried out under contract with the Committee on Food Research of the Quartermaster Food and Container Institute for the Armed Forces. The opinions and conclusions expressed are those of the authors, and are not to be construed as necessarily reflecting the views or endorsement of the War Department.

[b]Part of a thesis presented by one of us (HHB) to the Graduate Faculty of the University of Pittsburgh in partial fulfillment of the requirements for the degree of Doctor of Philosophy. Contribution No. 817, from the Department of Chemistry, University of Pittsburgh.

[c]Presented at the Tenth Annual Meeting of the IFT., Chicago, Illinois, May 24, 1950.

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following amines in 0.1 *N*. HCl; glycine, DL-alanine, n-butyl amine, aniline, p-toluidine, p-nitroaniline, and p-chloroaniline. The solutions were shaken, then tightly stoppered in test tubes and placed in an oven at 60°C. for five hours. At the end of this period the tubes containing the amino acids were very darkly colored and had begun to froth due to evolution of a gas. Of the simple amines, only n-butyl amine had browned as extensively as the amino acids. Solutions of p-toluidine and aniline were definitely, though less brown; the p-chloroaniline solution had become only slightly colored; and no change was noted with p-nitroaniline.

The above solutions were allowed to stand for three days at 60°C. At the end of this time all had developed very dark colors, accompanied by precipitation of dark colored solids. Ether was added to each and the layers permitted to separate. In the case of aniline and p-toluidine a slight brown color was observed in the ether layer. Otherwise the color remained entirely in the aqueous phase.

Preparation of glycosylamines. D-Glucose was condensed with a variety of amines by the procedure of Mitts and Hixon *(9)* and Kuhn and Birkofer *(4)*. Equimolar (0.05 *M*.) quantities were used in all condensations since it was found that an excess of amine promoted browning of the mixture at an accelerated rate. Excess glucose was difficult to separate from the condensation product. Although the conditions of condensation were essentially anhydrous, traces of water did not complicate the reaction except in those cases where the condensation products were extremely susceptible to browning. Anhydrous methanol, previously refluxed in contact with and then distilled from solid NaOH, was used as a solvent. The catalyst was a trace of HCl, NH_4Cl, or amine hydrochloride, only the least basic of the amines requiring the use of free HCl. All condensations were carried out at reflux temperatures. In most cases solidification of the reaction mixture to a mass of crystals signified completion of the reaction. The product was repeatedly recrystallized from methanol. The results are summarized in Table 1.

TABLE 1

Yields and decomposition points of glucosylamines

Code No.	D-Glucosyl derivative of	Yield	Decomposition point
		%	°C.
I	n-Butyl amine	75	96-98
II	Aniline	79	146-147
III	p-Chloroaniline	86	159-160
IV	o-Chloroaniline	42	151-153
V	3,5-Dichloroaniline	43	174-175
VI	2,4-Dichloroaniline	59	165-167
VII	p-Nitroaniline	93	183-184
VIII	p-Phenetidine	57	115-118
IX	o-Aminobenzoic acid	53	130-135

The N-glucosides IV, V, and IX could not be obtained entirely free of glucose. D-Glucosyl-m-chloroaniline, D-glucosyl-2,5-dichloroaniline, and D-glucosylcarbethoxyaniline browned so rapidly that they could not be isolated, while the glucose could not be made to condense with 2,4,6-tribromoaniline.

All attempts to condense D-glucose with p-nitroaniline or amines other than the above either resulted only in browning or recovery of the unchanged reactants.

Browning qualities of glucosylamines. One percent aqueous solutions of the glucosylamines were browned by heating at 60°C. The order of diminishing susceptibility to browning of the various amine derivatives is shown in Table 2.

2,3,4,6-Tetramethyl-D-glucosylamines. 2,3,4,6-Tetramethyl-D-glucosylaniline was prepared by condensing aniline with 2,3,4,6-tetramethyl-D-glucose by heating 0.01 *M*. quantities of the reactants at 130°C. for 30 minutes without solvent or catalyst. The cooled solid was taken up in 5 ml. of hot methanol and cooled, giving 1.50 g. (48%) of product melting at 136-138°C., as compared to a m.p. of 136-137°C. reported by Pringsheim and Schmalz *(12)*.

TABLE 2

Order of diminishing susceptibility to browning of D-glucosyl derivatives

1. n-Butylamine	5. p-Chloroaniline
2. p-Phenetidine	6. 3,5-Dichloroaniline
3. Aniline	7. 2,4-Dichloroaniline
4. o-Chloroaniline	8. p-Nitroaniline

2,3,4,6-Tetramethyl-D-glucosyl-p-chloroaniline was obtained in 58% yield by the above procedure. It melted at 146-148°C. as compared with a reported m.p. of 146-147°C. *(12)*.

2,3,4,6-Tetramethyl-D-glucosylglycine ethyl ester was prepared in an impure condition by warming equimolar mixtures of tetramethyl-D-glucose and freshly distilled glycine ethyl ester, prepared by the method of Fischer *(2)*, with small amounts of methanol. The latter was then removed in vacuo, leaving only slightly colored oils which were well washed with petroleum ether. In no instance could the product be induced to crystallize.

Observations on the browning of tetramethylglucosylamines. The tetramethylglycosylanilines proved to be soluble in all common solvents including water. They were unexpectedly resistant to browning, although in weakly acid solution some browning was evidenced on warming. The crude tetramethyl-D-glycosylglycine ethyl ester was found to be resistant to browning in anhydrous media, but browned very rapidly in the presence of water.

Observations on the browning of simpler carbohydrates. DL-Glyceric aldehyde was prepared from acrolein (Shell Development Company) by a series of reactions described in Organic Syntheses *(11)*. A 0.5% solution of the aldehyde and a 1.0% solution of D-glucose were prepared. Ten-ml. portions of these solutions were added to equal volumes of 1.0% solutions of glycine, n-butyl amine, DL-alanine, and aniline. The resulting mixtures were placed in stoppered test tubes and the browning observed at 50°C. In all cases the glyceric aldehyde browned more rapidly than did glucose in the presence of the same amine.

Glycolaldehyde free of the usual dimers and higher polymers was desired for investigation of the browning qualities of this simplest carbohydrate. A product reasonably free of impurities was obtained by decarboxylation of dihydroxymaleic acid, prepared from mesotartaric acid by the method of Neuberg and Schwenk *(10)*. From 100 g. of mesotartaric acid there was obtained approximately 10 g. of crude dihydroxymaleic acid which could not be further purified, but which was satisfactory for the subsequent conversion to glycolaldehyde.

Five g. of dihydroxymaleic acid was dissolved in 150 ml. of water and the solution warmed to 50°C. until the evolution of CO_2 ceased. The solution was then vacuum distilled and 100 ml. of the distillate collected in a flask immersed in an ice bath. The dilute solutions of glycolaldehyde thus obtained reduced Fehling solution in the cold. Their concentrations were determined by quantitative precipitation of the 2,4-dinitrophenylosazone from 5-ml. and 20-ml. aliquots. Each was treated with 1 g. of 2,4-dinitrophenylhydrazine in 15 ml. of glacial acetic acid. The solutions were boiled for 10 minutes under reflux, cooled and filtered through weighed sintered glass crucibles. The voluminous osazone was washed with alcohol and ether, then dried in vacuo over P_2O_5 and weighed. Without further purification the osazone melted at 218-320°C. Microanalysis for nitrogen:

Calculated for $C_{14}H_{10}O_8N_8$: N, 26.75%
Found: N, 26.50%.

Concentrations between 0.012 and 0.1 *M*. were obtained in various preparations of glycolaldehyde solutions, depending on the volume of distillate collected.

Solutions of glycolaldehyde and of glucose (both 0.05 *M*.) were made 0.5 *M*. in glycine, in DL-alanine, and in n-butyl amine. The solutions were tightly stoppered in test tubes and warmed at 50°C. It was observed that the glycolaldehyde solutions developed color much more rapidly than did those containing glucose, but that after 24 hours all the glucose solutions contained precipitates while none of the glycolaldehyde solutions had precipitated.

In a similar study it was found that glycolaldehyde solutions browned at approximately the same rate as did DL-glyceraldehyde solutions of the same molar concentrations.

Glycolaldehyde ethers. A series of glycolaldehyde ethers was prepared from bromacetal obtained by a modification of the method of Wislicenus *(13)*.

Ethoxyacetaldehyde, the ethyl ether of glycolaldehyde, was obtained in an impure condition from its acetal which was prepared by the procedure of Leuchs and Geiger *(7)*. Hydrolysis of the acetal gave a product which could not be obtained pure owing to the susceptibility of the ether linkage to hydrolysis. For this reason it did not effectively inhibit the browning reaction in the presence of amines and amino acids.

Phenoxyacetaldehyde was prepared from anhydrous potassium phenoxide (0.20 moles) and bromoacetal (0.25 moles) by heating on an oil bath at 180°C. with stirring for two hours. After washing with water and drying, the reaction mixture was distilled through a short column to give 13.5 g. of phenoxyacetal boiling at 127-130°C. at 11-mm. pressure. This was hydrolyzed with 5% sulfuric acid to the aldehyde by the method of Autenrieth *(1)*. The yield from 10.5 g. of the acetal was 5.3 g., or 51% of the aldehyde, boiling at 89-92°C. at 10-mm. pressure. Phenoxyacetaldehyde (X) is unstable and readily undergoes ring closure to coumarone. For storage it was converted into the stable bisulfite addition compound.

o-Chlorophenoxyacetaldehyde (XI), m-chlorophenoxyacetaldehyde (XII), p-chlorophenoxyacetaldehyde (XIII), and p-cresoxyacetaldehyde (XIV) were also prepared by the above method, and were converted to 2,4-dinitrophenylhydrazones for analysis. The yields and properties of the aryloxyacetals and the corresponding aldehydes are summarized in Tables 3 and 4.

TABLE 3

Synthesis of aryloxyacetals

Acetal of*	Boiling point	Yield	Microanalysis	
			Calculated	Found
	°C.	%	%	%
XI	142-144 at 7 mm.	55	Cl, 14.52	14.15
XII	155-158 at 12 mm.	86	Cl, 14.52	14.51
XIII	144-146 at 7 mm.	49	Cl, 14.52	14.20
XIV	132-135 at 12 mm.	40	C, 69.61	69.31
			H, 8.99	8.80

*See text for details.

TABLE 4

Synthesis of aryloxyacetaldehydes

Aryloxyaldehyde[f]	Boiling point	Yield based on		2,4-Dinitrophenylhydrazone		
		Bromo-acetal	Aryloxy-acetal	Melting point	Microanalysis for N	
					Calculated	Found
	°C.	%	%	°C.	%	%
XI	150-152 at 7 mm.	47	85	156-157	15.97	16.29 16.32
XII	148-150 at 13 mm.	78	91	142-143	15.97	16.12 16.29
XIII	149-151 at 13 mm.	43	88	129-130	15.97	16.12
XIV	136-138 at 14 mm.	36	90	144-145	17.15	17.15 17.45

[f] See text for details.

Browning of the aryl ethers of glycolaldehyde. Because of the tendency of the variously substituted phenoxyacetaldehydes to undergo ring closure to coumarones, the bisulfite addition compounds were used in the investigation of their browning susceptibilities. Since bisulfites as well as SO_2 are known to inhibit the browning reaction, it is likely that the marked resistance of these aldehydes to browning was in part due to

the presence of the bisulfite. However, no means of completely obviating this situation could be found.

Some 0.1 *M.* solutions of the bisulfite addition compounds of each of the above aryloxyacetaldehydes were prepared in 25% alcohol, and 10-ml. portions of each solution were added to equal volumes of a 1 *M.* solution of glycine in 0.1 *M.* HCl. Sulfur dioxide evolved from the solutions, which were placed in unstoppered test tubes in an oven at 75°C. After 24 hours color had developed in all the tubes, with the following gradation from maximum to minimum intensity: p-cresoxyacetaldehyde, phenoxyacetaldehyde, p-chlorophenoxyacetaldehyde, m-chlorophenoxyacetaldehyde, and o-chlorophenoxyacetaldehyde.

The solution containing p-cresoxyacetaldehyde was much darker than any of the others, while the difference between the solutions of p-chlorophenoxyacetaldehyde and its m-isomer was very small. The o-chlorophenoxyacetaldehyde solution had browned only very slightly.

The experiment was repeated with a 1 *M.* solution of glycine in 0.1 *M.* NaOH. After 24 hours at 75°C. all of the solutions had developed colors comparable with that of p-cresoxyacetaldehyde in acid solution. No definite gradation of color was noted, but in general the solutions were darker than the corresponding acid solution.

DISCUSSION OF RESULTS

From the results of the foregoing studies, some generalizations can be made regarding the ability of various amines to promote the browning reaction. Of particular importance are the basicities of the amines shown in Table 5 *(6)*. No data are available on the basicities of the more highly halogenated anilines, but presumably they are less basic than aniline.

TABLE 5

Basicities of various amines[a]

Amine	$K_b^{25°}$
n-Butyl amine	4.09×10^{-4}
p-Phenetidine	1.79×10^{-9}
Aniline	3.82×10^{-10}
p-Chloroaniline	8.45×10^{-11}
o-Chloroaniline	3.75×10^{-11}
m-Chloroaniline	2.88×10^{-11}
Ethyl p-aminobenzoate	2.30×10^{-12}
p-Nitroaniline	1.24×10^{-13}

[a] From reference *(6)*.

Comparison of the order of diminishing basicities with the order of diminishing effectiveness in promoting the browning reaction (Table 2) reveals that with the exception of o-chloroaniline a direct correlation exists. The increased susceptibility to browning of o-chloroaniline over its p-isomer is probably due to the steric effect of the chlorine in the o-position. This effect may also explain why 2,4,6-tribromoaniline cannot be condensed with glucose. That glycosyl-m-chloroaniline could not be prepared while its p-isomer was readily obtained, cannot be explained in terms of relative basicities. A partial explanation may lie in the fact that the p-isomer exhibits a weak mesomeric effect which is not shown by m-halogenated anilines. 2,5-Dichloroaniline, which likewise could not be condensed with glucose, exhibits both the o-steric effect and the peculiar phenomenon caused by an m-substituted chlorine.

From the work of Mitts and Hixon *(9)* on rates of hydrolysis, and observations reported in this paper it is seen that the glucosyl derivatives of the more basic amines and o-substituted anilines are the most highly dissociated in aqueous solution. These same amines were found to promote the most rapid browning. It is therefore concluded that the browning process does not involve actual condensation of the carbonyl group of the carbohydrate with the amine, but occurs by some other mechanism. Those amines which yield condensation products resistant to hydrolysis do not readily cause browning.

The rapid browning resulting from the use of glyceric aldehyde and glycolaldehyde indicates that furfurals are not necessarily intermediates in the browning reaction. However, the inhibitory effect of etherification of the hydroxyl group of glycolaldehyde and the similar effect observed with 2,3,4,6-tetramethylglucose indicate that the α-hydroxyl group of the carbohydrate is essential to the browning process. This conclusion is in agreement with the findings of Hurd and Kelso *(3)* who noted that 2,3,4-tridesoxyaldopentose did not exhibit browning in the presence of glycine, while 3,4-didesoxyaldopentose browned more readily than glucose.

The ethyl ether of glycolaldehyde was more easily hydrolyzed than the aryl ethers and resulted in more rapid browning. Browning of the aryloxyacetaldehydes in acid solution was attributed to the slow hydrolysis to glycolaldehyde and a phenol, the former then undergoing the usual browning reaction. Evidence of this is found in comparison of the relative rates of browning of the aryl ethers with the ionization constants of the corresponding phenols given in Table 6 *(5)*. Since the mechanism of hydrolysis

TABLE 6

Dissociation constants of certain phenols[b]

Phenol	$K_a^{25°} \times 10^{-11}$
p-Cresol	0.67
p-Chlorophenol	4.68
m-Chlorophenol	19.5
o-Chlorophenol	31.4
Phenol	1.23

[b] From reference *(5)*.

of ethers involves proton attack on the oxygen, ethers of less acidic (more basic) phenols are the more readily hydrolyzed. Thus the rate of hydrolysis of the ethers is directly proportional to the basicity of the oxygen atom and inversely proportional to the acidity of the corresponding phenol. Among the examples studied this relationship holds with the single exception of o-chlorophenoxyacetaldehyde. The discrepancy in the latter case is probably due to the steric effect of the o-substituted chlorine which inhibits hydrolysis of the ether.

The accelerated browning which occurred when the amine-aryloxyacetaldehyde reaction was carried out in basic solution remains unexplained. However, it agrees with the results obtained with other carbohydrate derivatives, in that browning always occurs more rapidly in basic than in acidic medium.

SUMMARY

A study was made of the reactions of a series of primary aliphatic and aromatic amines with a number of simple carbohydrate derivatives. Included among the latter were D-glucose, 2,3,4,6-tetramethyl-D-glucose, glycerie aldehyde, and glycolaldehyde together with its ethyl, phenyl, and several substituted phenyl ethers. The more strongly basic amines whose glycosyl derivatives are the most highly dissociated in aqueous solution, are more effective than others in promoting browning. D-Glucose, glycerie aldehyde, and glycolaldehyde exhibited great susceptibility to browning, increasing in the order named. Tetramethylglucose and the glycolaldehyde ethers proved resistant to browning in proportion to their resistance to hydrolysis. Thus the browning reaction does not involve actual condensation of the amine with the carbonyl group of the carbohydrate, and the formation of furane derivatives is not essential to browning. However, the α-hydroxyl group of the carbohydrate is essential.

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AEROBACTER AEROGENES AS A CAUSE OF ROPY BRINE IN CUCUMBER FERMENTATION[a]

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Ropy fermentation is encountered frequently in the food industry. The cause of ropiness, as found by many investigators, has been bacteria.[b] The present study was conducted in August of 1950 in vats of fermenting cucumbers in Michigan. Brine in some, but not all, of the vats at this salting station had turned ropy and was causing considerable anxiety to the salter. He had not encountered this particular condition before and wanted to know what caused it.

ISOLATION AND IDENTIFICATION

Ropy brine was collected from the vats and 0.1 and 1.0 ml. quantities were first seeded into an enrichment medium consisting of nutrient broth to which had been added one percent yeast extract. These tubes were incubated at 30°C. for 48 hours, after which the supernatant liquid was poured off. The ropy mass left at the bottom was placed in a dilution bottle which was shaken for five minutes or more in a shaking machine oscillating 180 times per minute. One ml. from this dilution blank was then placed in another 99-ml. dilution bottle and it in turn was shaken for five minutes or more. This procedure was repeated until sufficient serial dilutions were obtained to insure distinct colonies on a plate. Microscopic examination of the dilution water showed distinct cells in most cases after the five-minute shaking. One ml. from the serial dilution blanks was plated on Difco tryptone glucose extract agar (T.G.E. agar). The plates were incubated for 24 hours at 30°C. after which distinct colonies were fished from the plates into the enriched nutrient broth. This broth was incubated for 48 hours at 30°C., the supernatant liquid decanted, and the ropy mass poured into a 99-ml. dilution blank which was shaken as previously described. Serial dilutions were made from this first dilution and shaken five minutes, and one-ml. quantities plated from the dilutions into T.G.E. agar. This cycle was repeated seven times until it was certain that the colonies were growing from a single cell.

From the many colonies thus obtained, six typical colonies were chosen for identification. A Gram stain, made from 24-hour cultures grown on T.G.E. agar slants, showed singly occurring, gram negative rods having a size of 0.6 to 8 x 1.2 to 1.9 microns. A capsule stain showed them to have distinct capsules. Colonies on nutrient agar after 24 hours were thick, glistening, raised, opaque, slightly elevated, and had a convex surface with

[a] Journal article No. 1262.

[b] For a list of foods in which ropy fermentation was observed and for a literature survey of this subject the reader is referred to a previous paper *(1)* from this department.

a mucoid consistency. The colonial margin was smooth and entire. The subsurface colonies were disc shaped.

The biochemical reactions of the organisms were as follows:

Reaction on:	Isolate					
	1	2	3	4	5	6
Glycerol 0.5%	AG	AG	G	G	G	G
Glycerol 1.0%	AG	AG	AG	AG	AG	AG
Dulcitol	AG	AG	AG	AG	AG	AG
Xylose	AG	AG	AG	AG	AG	AG
Mannite	AG	AG	AG	AG	AG	AG
Lactose	AG	AG	AG	AG	AG	AG
Maltose	AG	AG	AG	AG	AG	AG
Sucrose	AG	AG	AG	AG	AG	AG
Dextrose	AG	AG	AG	AG	AG	AG
Salacin	AG	AG	AG	AG	AG	AG
Lauryl Tryptose	G	G	G	G	G	G
Gelatin plates, *(2)*	—	—	—	—	—	—
Litmus milk	AC	AC	AC	AC	AC	AC
Indol	+	+	+	+	+	+
Methyl red	—	—	—	—	—	—
Voges-Proskauer	+	+	+	+	+	+
Kosher citrate	+	+	+	+	+	+

The symbol AG indicates acid and gas, G indicates gas, AC indicates acid and coagulation, + indicates positive reaction, and — indicates negative reaction.

None of the colonies produced H_2S on Kliger's iron agar but produced an acid butt and slant on this medium. Characteristic mucoidal colonies were produced on Levine's E.M.B. agar.

Several interesting observations were made during the study. One observation was that ropiness occurred in most sugar media but in different amounts. The largest amount of ropiness occurred in dextrose and lauryl tryptose broths, followed by sucrose, lactose, and maltose broths. In other sugar and alcohol broths the ropiness was slight or absent.

SUMMARY

From several tanks of ropy pickle brine the predominating organism isolated was *Aerobacter aerogenes* according to the biochemical, cultural, and morphological characteristics of the group studied.

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SOME ASPECTS OF MICROBIOLOGY APPLIED TO COMMERCIAL APPLE JUICE PRODUCTION. I. DISTRIBUTION OF MICROORGANISMS ON THE FRUIT

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Although much has been written on the technology of apple juice production, its microbiological aspect has apparently received but scant attention. When apple juice is stored in bulk for many months prior to bottling, some form of strict microbiological control is essential if growth of spoilage organisms is to be suppressed. This is of especial importance in any factory where the Boehi method of storing juice in bulk under carbon-dioxide pressure is installed, a system which has been widely adopted in Western European countries.

Accordingly, some four years ago, we commenced a series of investigations at an apple juice factory in Gloucestershire for the purpose of developing a system of control applicable to apple juice production. The original investigation has since been extended to embrace a complete study of the microbiology of apple juice. Certain aspects of this investigation, we feel, may prove of general interest to microbiologists engaged in both the apple juice and cider industries and accordingly, they will be presented in this series of papers.

From the microbiological point of view, the primary consideration in the production of high quality apple juice lies in the initial soundness of the fruit. This will determine the number of spoilage organisms to be found in the freshly expressed juice provided that chances of contamination from external sources are reduced to a minimum.

Very few attempts to assess the magnitude of the microflora population of fruit have been reported. Pederson *(3)*, in experiments with Concord grapes, found that after stemming and pulping the total number of yeasts varied from 60,000 to 1,200,000 per cc. of pulp. Forgacs *(2)*, using a swabbing technique on selected areas of the cuticle, found that unwashed apples contained on the average 252 mesophilic and 6 thermophilic microorganisms per 4 square inches of surface. These counts, particularly of the mesophilic organisms, are exceedingly small and this may be due to the high incubation temperature (37°C.) that he employed.

On arrival at the processing plant, apples invariably contain varying proportions of rots and, despite careful sorting, it is impossible to eliminate these completely. Slightly damaged and bruised fruit can be safely used for apple juice manufacture but must be expected to bear varying numbers of microorganisms. It seemed desirable, therefore, that our preliminary investigations should be devoted to assessing the number of microorganisms to be found associated with the fruit. Compilation of such data over several pressing seasons would serve a two-fold purpose. First, it would assist the plant microbiologist to determine the extent to

which the additional contribution to the microbial population from damaged fruit can be permitted without unduly increasing the hazards of spoilage in the expressed juice. Second, suitable utilization of the data would also prove invaluable as a control measure to check the efficiency of the fruit grading department.

We shall describe in this paper the methods we have developed for assessing the numbers of organisms found present on fruit grown in the West of England in the apple orchards of Gloucestershire. The significance and interpretation of the data obtained will also be discussed. It is to be regretted that, apart from the results recorded by Forgacs, no records for counts on apples from other fruit growing areas are available for comparison.

METHODS

Sampling is a matter of some difficulty, for differentiation between sound and unsound fruit is not easy when one realizes that the degree of rot on unsound fruit varies in intensity from a complete visible rot to that of a minute epidermal infection invisible to the naked eye.

Batches of fruit were selected in which the apples appeared either visually sound or else infected to a similar degree. In order to raise the accuracy of our results, counts were made on a number of apples chosen at random from each batch, the number of replicates varying from three to twenty and an average microbiological count estimated per apple. It will be readily appreciated that in an investigation of this nature close agreement between replicates cannot be anticipated, even on sound fruit where counts may be confined to the cuticle, since the organisms deposited there are the result of chance external infection. However, as will be demonstrated, the average counts, estimated from replicate experiments, have been found to possess some considerable significance.

Assessment of Microbial Counts

We have devised methods for assessing total counts on a, the entire surface of the cuticle or epiderm; and b, on any selected part of the fruit or on the entire apple. The first method is the least accurate for it involves an estimation of the total external surface area of the fruit. The method is only of particular value for comparing counts on apples of varying size, the counts being expressed in terms of "population densities" which represent the average number of microorganisms per cm.^{2} of surface.

The simplest method of calculating an approximation of the surface area is to measure the greatest diameters of the fruit in both vertical (d_1) and horizontal (d_2) planes. Then the mean radius equals $(d_1 + d_2)/H$ and the surface area equals $4\pi[(d_1 + d_2)/4]$.

We have checked the reliability of this approximation from estimations of the surface area calculated from the volume displacement of apples in water. Calculations by both methods on individual apples yielded excellent agreement.

Swabbing technique. This can be employed only for estimating organisms on the cuticle or epiderm and it is first necessary to calculate the surface area as described above.

For carrying out the swabbing, swabs are prepared from cotton wool wound on a stiff wire and previously sterilized in a test tube by dry heat. The apple is held with large sterile surgical tissue forceps and swabbed first with a dry swab. This is then thoroughly wetted and washed in a test tube with 10 ml. of sterile water. Next the surplus water is squeezed out by pressing the swab against the inner surface of the tube and the swab is again used to sweep the cuticle. This is repeated twice. The swab is then teased apart with forceps and the cotton wool placed in the tube of sterile water.

Another dry swab is then applied and the whole surface of the apple swabbed as before, the swab eventually being again teased apart and mixed with the sterile water. The entire contents of the tube are then thoroughly agitated to ensure uniform distribution of the microorganisms. Dilutions in sterile water are made using the entire volume of 10 ml. of liquid. Subsequent dilutions follow, using a pipette with an enlarged delivery orifice to allow cotton wool fibres to be drawn up. The final dilutions are plated out on apple juice agar and incubated.

The plate colonies are finally counted and after correcting for dilution are expressed either as the number of organisms per total surface area or as population densities in terms of number of organisms per cm.^{2} of epiderm.

Pulping technique. This is less restricted in its application than the swabbing technique and can be usefully employed for the following purposes:

1. Estimation of the total number of microorganisms per whole apple or average number per unit weight of whole apples.
2. Estimation of the total number of microorganisms in any specified part of the fruit (flesh or core).
3. Assessment of the number of microorganisms on the epiderm of a sound apple, either as a total or in terms of "population density."

TABLE 1

Comparison of the swabbing and pulping techniques for assessing the population densities of the microflora on apples

Population densities per cm.^{2} epiderm					
Yeasts		Lactic acid organisms		Acetic acid organisms	
Swabs	Pulping	Swabs	Pulping	Swabs	Pulping
1	2	2,100	1,659	425	671
71	98	995	1,433	431	669
1,550	2,200	17	14	15	20
650	851	9	6	25	41
94	56	37	83	36	44
80	130	54	76	12	8
42	81	103	154	46	24
105	235	340	390	20	75

The technique is as follows: After weighing, the sample is sliced aseptically, or for epidermal counts the latter is carefully removed in a thin layer with a sharp sterile knife. The tissue, after weighing, is then pulped in a sterile glass beaker by grinding with a sterile glass rod flattened out to a disc at the lower end. A measured volume (up to 100 ml.) of sterile water is added and the grinding continued until a homogeneous suspension is obtained. The aqueous suspension of pulp is then carefully weighed, aliquots removed, and a series of dilutions in sterile water prepared. After thorough shaking, 1-ml. quantities of the final dilutions are withdrawn and plated out on apple juice agar, and incubated for 48 hours at 25°C.(77°F.).

Plating-out media. Both dextrose agar and apple juice agar are useful media for plating out and are equally effective. Apple juice agar may be prepared by diluting apple juice sufficiently to reduce its sugar content to 1 to 2%. To 500 ml. of diluted juice 1 g. ammonium phosphate and 10 g. of powdered agar are added, and the mixture is heated in a steam sterilizer until dissolved. It is then filled into flasks and tubes and sterilized in the usual manner. If the acidity of the apple juice is high, it may be necessary to increase the proportion of agar to obtain a rigid gel.

Comparison of swabbing and pulping techniques. Many experiments were carried out to compare both techniques and it was found that agreement between each was sufficient for comparative purposes for the investigation under study. Some typical results are shown in Table 1. Apples were halved aseptically, yeasts, acetic acid, and lactic acid organisms were estimated on one half by the pulping technique and on the other half by swabbing. Theoretically, there should be no gross difference between the two methods. For ease of manipulation, swabbing is the better method but accuracy will depend on the skill required to remove all organisms without redeposition on the surface that is being examined. Accuracy by the pulping technique, when applied to epidermal counts, will depend on the soundness of the fruit, for if the epiderm be ruptured and infection of the underlying tissues has taken place, the method becomes useless.

SEASONAL CHANGES IN THE MICROORGANISM COUNT

As a matter of interest we commenced our investigations by examining whether the microflora population exhibited any change in numbers during growth and maturity of the fruit. If changes of considerable magnitude occur during the period of maturation, obviously the time of gathering will have an important bearing on the degree of contamination expected.

TABLE 2

Seasonal variations in population densities of the microflora present on the epiderm of Grenadier (scab-resistant) apples

Date	Average number of organisms per cm.² epiderm			Temperature and rainfall			
	Yeasts	Lactic acid	Acetic acid	Temp. °F.		Rainfall, inches	Total rainfall per month, inches
				Max.	Min.		
June 8	2	2,100	390	59	54	0.01	
20	.....	980	1,500	60	45	.....	
28	7	3,500	900	83	60	.....	0.44
July 12	2	3,500	900	57	64	.....	
25	8	1,470	1,500	87	61	.....	
28	5	800	3,900	80	51	.....	1.51
Aug. 7	20	495	800	72	56	.....	
14	160	1,700	780	78	52	.....	
22	60	530	920	83	60	.....	
29	700	130	150	75	57	.....	1.48
Sept. 2	470	170	700	77	51	.....	
9	3,600	90	120	66	64	.....	
16	5,000	120	100	57	49	0.01	
22	3,500	40	80	67	56	0.27	
29	2,100	20	50	68	58	.....	1.96
Oct. 3	400	60	10	67	54	.....	
9	500	80	20	62	56	0.38	
14	100	190	70	66	48	.....	
17	300	570	40	63	50	0.33	
22	820	640	140	55	45	0.13	6.62

For the purposes of this investigation a tree of scab-resistant Grenadier apples was selected and samples of fruit were gathered over the period from June to October. The apples were gathered at random from different parts of the tree and counts of yeast, acetic acid, and lactic acid organisms were made by pulping the peeled epiderm. Acetic acid and lactic acid organisms were differentiated by Gram's staining methods and by the catalase reaction described by Tosic and Walker *(5)*. Unfortunately, it was not possible to enumerate the molds at the time this experiment was carried out. The results are given in Table 2.

The yeast counts were exceedingly low at the commencement of observations, and after attaining a maximum population density of 5,000 per cm.² in mid-September showed a marked decrease at the beginning of October. The acetic and lactic acid organisms were present in large numbers in June and thereafter, the population densities decreased until the

end of October, when a slight increase was noted. Throughout the entire period of observation until the end of September, the weather was warm, sunny, and dry, so there was no possibility of fluctuation of population densities being due to major changes in climatic conditions. It is apparent, therefore, that despite the influence of wind and rain, the number of micro-organisms on the fruit will vary according to the season when the fruit is harvested.

DISTRIBUTION IN DIFFERENT PARTS OF THE FRUIT

Barker *(1)* has stated that the presence of yeasts on the surface of the apple has been traced as far back as the time of opening of the blossom, and Tanner *(4)*, has remarked that several workers have reported the presence of microorganisms in the inner structures of various fruits, seeds, and plant tissues as well as in the sap. We had to consider, therefore, that even in perfectly sound fruit, localization of the microflora might not be confined to the epiderm, in which case neither epidermal swabs nor pulping would provide a reliable guide to the total number of organisms per whole fruit.

Accordingly, we selected a batch of sound Bramley Seedling apples from which the peels were removed aseptically and as thinly as possible, special care being taken to avoid the brushing off of any microorganisms. Each apple was then halved and the cores also removed aseptically. Epiderm, flesh, and core were then each pulped separately. The results given in Table 3 are the averages of replicate determinations. These results demonstrate that although no yeasts are found in the flesh in sound fruit, they are present in the core as well as on the epiderm, but to a lesser extent.

TABLE 3

Distribution of yeasts in sound Bramley Seedling apples

Section of fruit	Total yeast count	Yeast count per g. of sample
Whole apple	1,150,000	13,988
Epiderm	1,195,000	12,892
Flesh	0	0
Core	5,507	790

FRUIT WITH INTERNAL ROT AROUND THE CORE

Several batches of apples appearing quite sound externally revealed on sectioning, areas of rot localized around the core and extending for varying distances into the flesh. Using aseptic precautions, these apples were sectioned and the infected areas completely removed. Portions of the surrounding tissue which microscopically appeared quite sound were also sampled. It was impossible, however, to be certain that the latter were being taken from areas free from yeast cell infection. Yeast and mold counts were made and the results are shown in Table 4.

The fungal micro-flora of the core comprised: *Penicillium expansum*, 74%; *Monilia*, 20%; and *Mucor mucedo*, 6%. The yeast counts on the cores of these apples were high compared with the numbers found in the

TABLE 4

Distribution of yeasts and molds in Bramley Seedling apples with internal rot

Core and surrounding areas of rot. Weight examined: 28.35 g.		
Total yeast count	Yeasts per g.	Molds per g.
830,000	29,276	15,300
640,000	22,576	23,800
Portion of pulp not showing rot and apparently sound. Weight examined: 28.35 g.		
Total yeast count	Yeasts per g.	Molds per g.
3,000	106	0
8,000	282	0

TABLE 5

Distribution of yeasts and molds in Bramley Seedling apples exhibiting *Penicillium expansum* rot

Epiderm		Pulp including core
Total yeast count	Yeast count per cm.²	Total yeast count
6,000,000	84,649	36,000,000
9,410,000	116,173	27,500,000
8,500,000	83,089	.....
Total mold count	Mold count per cm.²	Total mold count
725,000,000	9,200,000	98,500,000
56,500,000	760,000	120,000,000
420,300,000	5,250,000	4,000,000

TABLE 6

Yeast and mold population of sound apples

Yeast counts			Mold counts		
Apple variety	Total organisms per epiderm	Organisms per cm.² epiderm	Apple variety	Total organisms per epiderm	Organisms per cm.² epiderm
Mixed culinary	1,150,000	.....	Mixed culinary	4,800	59
Mixed culinary	11,200	89	Mixed culinary	16,000	226
Mixed culinary	4,300	34	Mixed culinary	3,500	61
Mixed culinary	9,300	74	Mixed culinary	1,400	12
Newton Wonder	1,600,000	20,400	Mixed culinary	25,000	288
Severn Bank	70,000	1,200	Mixed culinary	7,300	83
Blenheim Orange	39,400	270	Kingston Black	10,000	85
Bramley Seedling	1,340,000	12,889	Kingston Black	110,000	873
Bramley Seedling	1,050,000	12,961	Bramley Seedling	14,000	.....
Keswick	270	3	Mixed cider fruit	9,400	116
Keswick	6,760	170	Mixed cider fruit	19,700	344
Mixed cider fruit	151,400	3,750	Mixed cider fruit	4,300	47
Mixed cider fruit	67,570	1,501	Mixed cider fruit	28,500	224
Mixed cider fruit	19,150	340	Mixed cider fruit	140,000	1,730
Kingston Black	6,020	150	Mixed cider fruit	7,500	92
Kingston Black	9,415	211	Mixed cider fruit	12,000	170
Grenadier	109	2	Mixed cider fruit	15,300	212

cores of sound fruit, and infection had also spread to areas of the flesh that visually had appeared perfectly sound.

It was considered that this localized internal infection might be due either to:

1. Penetration by insects acting as yeast carriers. This has been known to occur but the fruit used in this experiment showed no obvious signs of penetration.

2. At the blossom stage, the interior of the gynaecium may become contaminated with yeasts and molds from either airborne infection or insect carriers during pollination. During formation and growth of the fruit, the microorganisms become sealed and it is assumed that under certain conditions when the apple approaches maturity and the acidity decreases, these molds are able to penetrate the tissues of the core and extend their growth into the flesh. After the internal tissues have been broken down by the fungi, the yeasts can then develop and spread to surrounding areas.

TABLE 7

Yeast and mold population of unsound fruit

Yeast counts		Mold counts	
Apple variety	Total organisms per epiderm	Apple variety	Total organisms per epiderm
Unknown, with blue mold infection	58,796,713		1,600,000
Unknown, with blue mold infection	45,858,607	Unknown varieties infected to varying degree with blue mold rot	201,000
Unknown, with blue mold infection	38,694,000		18,610,000
Kingston Black, with blue mold infection	64,400,000		9,260,000
Blenheim Orange	21,400,000		218,500,000
Bramley Seedling	42,000,000		725,000,000
Bramley Seedling, with internal core rot	1,000,000		56,500,000
Bramley Seedling, with internal core rot	1,300,000		420,300,000

FRUIT WITH BLUE MOLD ROT

This was a typical *Penicillium expansum* rot. Examination of the fruit was confined to the epiderm and to the flesh inclusive of core, since demarcation between infected areas of the core and flesh in such fruit is difficult to define. The Bramley Seedling apples examined were in a very early stage of infection which was not visually apparent. As shown in Table 5, the exceedingly large numbers of yeasts present were much higher in the flesh than on the epiderm. It is apparent that once the cuticle is injured and mold infection develops, the interior of the fruit is very rapidly rendered favorable for development of the yeasts.

RANGE OF VARIATION IN EPIDERMAL COUNTS

We have already shown that large numbers of microorganisms may be found on perfectly sound fruit. It is of importance to estimate the range of variation in population that may be expected between different varieties of fruit and different consignments of the same variety as delivered at the processing plant. Some typical counts on sound fruit carried out over several pressing seasons are shown in Table 6. For comparative

purposes, disparity in apple size has been eliminated by also expressing the results in terms of "population densities."

Table 7 shows a selection of comparative data on fruit exhibiting various degrees of damage and mold infection. Here, owing to the physical condition of the fruit, surface area approximations proved impossible to determine, and hence results have only been expressed as total counts per apple.

DISCUSSION

Assessment of the distribution of the microflora on apples, particularly on sound fruit provided figures much higher than had been anticipated. Considerable significance can be attributed to the data that has been compiled and this will now be discussed.

Earlier in this paper we compared our swabbing and pulping techniques for assessing the yeast counts on fruits, from which it may be assumed that fairly reliable estimates of the yeast population may be obtained. In comparison, estimations of the mold population present some considerable difficulty. In fact, a statement that the mold population density of an apple is X organisms per cm.^{2} is virtually meaningless unless X be interpreted as being the number of individual colonies per cm.^{2} That as such is, however, incapable of determination. The only real value of mold counts is that they provide an approximate indication of the potential number of mold colonies that under favorable conditions could develop in the expressed juice from spores and regenerative segments of mold mycelium present on contaminated fruit. This is of more direct interest to the apple juice manufacturer than the knowledge of the actual number of mold colonies, were they capable of estimation on the fruit itself.

In view of what has been stated, the pulping technique will provide results more comparable with conditions prevailing in practice than those obtained by epidermal swabs, and a more effective distribution of spores can be obtained. Some difficulty may, however, be experienced with fruit that is heavily contaminated on account of the higher dilutions required to yield a plate count that can be readily assessed. It is impractical to estimate mold counts on plates containing more than five to ten colonies, and accordingly at high dilutions there will be an increase in error.

The population densities of the different groups of microorganisms on sound fruit have been found to exhibit enormous variation and also to fluctuate to a considerable extent on any one variety during growth and maturity on the tree. Organisms are not confined to the cuticle but are also invariably present in the core, but do not penetrate to the flesh unless there is either preliminary internal or external breakdown of the outer tissues by fungal infection. When tissue breakdown does occur, yeasts may invade the flesh and develop in areas that still remain free from mold growth.

In damaged fruit yeast and mold counts become fantastically high. Based on the data of Tables 6 and 7, one may attempt to define arbitrary limits for yeast and mold counts to differentiate between sound and unsound fruit. The total yeast counts of sound fruits varied from 100 to 1,600,000 per apple and the corresponding range for molds was 800 to

140,000. If these figures are compared with the counts given in Table 7, one may state that sound fruit may be expected to possess epidermal yeast and mold counts of up to 2,000,000 and 200,000 respectively. Fruit showing counts in excess of these figures is almost certain to be damaged, and should either the total yeast or mold count exceed 5,000,000, the fruit is unsuitable for production of apple juice. These limits must, however, be regarded as merely arbitrary, and applicable only to fruit grown in certain orchards of Gloucestershire, based on observations recorded mainly over a period of four years. Conditions elsewhere may prove to be widely different.

SUMMARY

A technique has been developed for assessing the number of microorganisms present on apples. It has been found that during growth and maturity on the tree, the yeasts and bacteria on the surface of the fruit undergo continual change in population density, even when variation in climatic conditions is at a minimum. In sound fruit, the presence of microorganisms is not confined to the cuticle but they are also present, to a lesser extent, in the core. Yeasts are not found in the flesh unless there has been either internal or external breakdown through fungal activity, when they may be found present in areas still free from mold contamination. Sound fruit exhibits considerable variation in yeast and mold counts. Apples with yeast or mold counts in excess of 2,000,000 and 200,000, respectively, may be assumed to be damaged and with counts in excess of 5,000,000 the fruit is unsuitable for apple juice manufacture. These limits are arbitrary only and are not necessarily generally applicable.

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